308

A STUDY OF HYDROGEN SULPHIDE PRODUCTION BY BACTERIA AND ITS SIGNIFICANCE IN THE SANITARY EXAMINATION OF WATER.

STANFORD UNIVERSITY LIBRARY MAR 28 1915

A THESIS

PRESENTED TO THE FACULTY OF THE GRADUATE SCHOOL OF CORNELL UNIVERSITY FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY.

BY

HARRY WESTFALL REDFIELD.

STANFORD UNIVERSITY LIBRARIES WITHDRAWN

ITHACA, N. Y.

1912.

3781 C81R UNCLASSED Dissertation

A STUDY OF

HYDROGEN SULPHIDE PRODUCTION BY BACTERIA AND ITS SIGNIFICANCE IN THE SANITARY EXAMINATION OF WATER.

A THESIS

PRESENTED TO THE FACULTY OF THE GRADUATE SCHOOL OF CORNELL UNIVERSITY FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY.

BY

HARRY WESTFALL REDFIELD.

ITHACA, N. Y.

1912.

FOREWORD.

The author desires to express his deepest gratitude to Professor E. M. Chamot at whose suggestion and under whose direction this work was carried out. To Professor L. M. Dennis, the head of the Department of Chemistry, and to Professor A. W. Browne, the author wishes to acknowledge his appreciation of their kindly interest in the work.

TABLE OF CONTENTS.

	PAGE.
FOREWORD	II
CHAPTER I. HISTORICAL REVIEW OF LITERATURE	1
Schardinger's work	2
Dunham's work	4
INTRODUCTION TO EXPERIMENTAL WORK	6
CHAPTER II. SPECIAL APPARATUS AND REAGENTS	7
Apparatus	7
Reagents	7
Sewage sample used for inoculations	8
CHAPTER III. EFFECT ON HYDROGEN SULPHIDE EVOLUTION PRODUCED BY FILTERING PEPTONE SOLUTIONS	9
Summary	10
CHAPTER IV. EFFECT ON HYDROGEN SULPHIDE EVOLUTION OF DIFFERENT CONCENTRATIONS OF PEPTONE	11
The triangular diagram	11
Results with peptone and sodium chloride	13
Two component diagrams	13
Peptone in presence of salts other than sodium chloride	15
Effect of the addition of beef extract to the medium	15
Summary	17
CHAPTER V. EFFECT ON HYDROGEN SULPHIDE EVOLUTION PRODUCED BY DIFFERENT INORGANIC SALTS	18
Modified use of triangular diagram	18
Check solutions used	19
Results with sodium chloride	20
Results with sodium sulphate	20
Results with potassium chloride	20
Results with magnesium chloride	21
Results with calcium chloride	21
Results with ammonium chloride	21
Results with sodium chloride, potassium chloride, ammonium chloride, calcium chloride, magnesium chloride, sodium sulphate, potassium sulphate, ammonium sulphate, calcium sulphate, magnesium sulphate, sodium nitrate, potassium nitrate, ammonium nitrate, calcium nitrate and magnesium nitrate	22
Results with primary phosphate	26
Results with secondary phosphates	27
Results with tertiary phosphates	29
Results with various combinations of potassium chloride, sodium chloride and potassium sulphate in conjunction with secondary potassium phosphate and secondary sodium phosphate	30
Results with calcium carbonate	31

Results with primary sodium carbonate 32
Effect of the inorganic salts contributed by the water under examination 33
Summary 34
CHAPTER VI. EFFECT ON HYDROGEN SULPHIDE EVOLUTION PRODUCED BY USING DIFFERENT BASES AND ACIDS IN ADJUSTING THE INITIAL REACTION TO PHENOL PHTHALEIN 37
Summary 37
CHAPTER VII. EFFECT ON HYDROGEN SULPHIDE EVOLUTION OF THE INITIAL REACTION OF THE MEDIUM TO PHENOL PHTHALEIN 38
Effect of temperature on reaction 38
Effect of salts on reaction 40
Optimum reaction for media 40
Summary 41
CHAPTER VIII. RELATION TO HYDROGEN SULPHIDE EVOLUTION OF THE FINAL REACTION OF THE CULTURES TO PHENOL PHTHALEIN 42
Effect of temperature on reaction 42
Deductions from final reaction 42
Note on the speed of evolution of hydrogen sulphide 45
Summary 46
CHAPTER IX. THE METHOD FOR DETECTING THE BACTERIA WHICH PRODUCE HYDROGEN SULPHIDE AS IT HAS BEEN FOUND TO GIVE MOST RAPID AND RELIABLE RESULTS 47
Summary 48
CHAPTER X. THE SENSITIVENESS AND RELIABILITY OF THE METHOD 49
With artificial sewage 49
With natural water supplies 50
Summary 55
CHAPTER XI. A COMPARATIVE STUDY OF METHODS FOR THE QUANTITATIVE DETERMINATION OF SULPHUR IN PEPTONE 56
Methods for total sulphur 56
Liebig method 56
Osborne method 56
A. O. A. C. method 57
Liebig-Koch method 57
Petersen method 60
Parr method 61
Methods for part of sulphur only 61
Schultz method 61
Nitric acid—potassium chlorate method 61
Hydrochloric acid—potassium chlorate method 62
Sulphur in untreated peptone 62
Extraction of peptone with alcohol 62
Total sulphur in alcohol extract 63
Total sulphur in residue from alcohol extraction 63
Summary 64

CHAPTER XII. QUANTITATIVE DETERMINATIONS OF SULPHUR IN THE CULTURE MEDIUM FOR THE DETECTION OF THE BACTERIA PRODUCING HYDROGEN SULPHIDE ---- 65
Sulphur in insoluble residue ---- 65
Peptone and sulphur used up by bacteria in flasks of different size 65
Effect of potassium chloride and of sewage on sulphur determinations ---- 66
Discussion of Table VI ---- 67
Effect of amount of surface exposed ---- 68
Discussion of Table VII ---- 71
Determinations of hydrogen sulphide ---- 71
Discussion of Table VIII ---- 73
Determinations of hydrogen sulphide with shorter incubation ---- 75
Discussion of Table IX ---- 75
Mercaptans ---- 77
Sulphur in alcohol extract of peptone and in residue from such extraction ---- 77
Hydrogen sulphide production from part of peptone insoluble in alcohol ---- 77
Discussion of Table X ---- 78
Hydrogen sulphide production from part of peptone soluble in alcohol ---- 80
Discussion of Table XI ---- 80
Callibration of blackening of lead acetate papers in necks of culture flasks ---- 82
Summary ---- 85
CHAPTER XIII. COMPARISON OF THE RAPIDITY OF HYDROGEN SULPHIDE PRODUCTION AND THE AMOUNTS PRODUCED WHEN ARTIFICIAL SEWAGES MADE FROM HUMAN FECES AND FROM THE FECES OF VARIOUS ANIMALS WERE USED ---- 88
Discussion of Table XIII ---- 89
Summary ---- 89
CHAPTER XIV. TESTS FOR HYDROGEN SULPHIDE PRODUCTION MADE WITH DIFFERENT STRAINS OF THE COLON ORGANISM ISOLATED FROM HUMAN FECES ---- 90
Summary ---- 92
CHAPTER XV. THE SIGNIFICANCE OF HYDROGEN SULPHIDE PRODUCTION IN THE SANITARY EXAMINATION OF WATER ---- 93
Summary ---- 96
ORGANISMS WHICH HAVE BEEN REPORTED AS PRODUCING HYDROGEN SULPHIDE ---- 97
GENERAL SUMMARY ---- 99
BIBLIOGRAPHY ---- 107

CHAPTER I.

HISTORICAL REVIEW OF LITERATURE.

The first record we find of hydrogen sulphide production by bacteria is in 1877 when M. U. Gayon[1] found this gas produced in eggs by micro-organisms, proving its presence by means of the black color of lead sulphide produced when the gas was brought in contact with lead acetate.

Two years later, in 1879, P. Miquel[2] announced the finding of an organism in "sewage, filthy water and drinking water", having the power of changing free and combined sulphur to hydrogen sulphide.

In 1882, J. Boehm[4] noted the production of hydrogen sulphide from well water and flowers of sulphur in the absence of air. He found that distilled water was not good to use in place of well water and claimed that it was the chalk and gypsum in the well water which favored the production of the hydrogen sulphide.

In the following year, F. Fischer[5] found 1.25% of hydrogen sulphide in the Paris sewers.

In 1884, C. Richet[6] worked on the "prohibiting power" of mercuric chloride, zinc chloride, cadmium chloride, copper chloride, ferric chloride, and nickel chloride on hydrogen sulphide production, using a medium consisting of sea water and .1% peptone, and inoculating with urine.

In 1886, Hoppe-Seyler[8] claimed to have obtained hydrogen sulphide from calcium sulphate by bacterial action. The amount of this gas obtained was measured by means of iodine.

In the same year, Hoertling[9] contributed a piece of work on the occurrence of hydrogen sulphide in urine, and in the following year, Mueller[11], [12] published on the same subject. He used lead acetate paper for testing and claimed that the source of the hydrogen sulphide was not in the albumin nor cystin, nor potassium sulphocyanide nor sulphate, but was in an unknown "mother substance".

This led to an animated discussion as to what was really the scource of the hydrogen sulphide and much work followed by Rosenheim[13], [14], Salkowski[15], Schrank[16], Holschewnikoff[17], Fromme[18], Debray and Legrain[19], Stagnitta and Balistreri[20],

Petri and Maasen[21], [22], [23], and Rubner[24], [25], between the years 1886 and 1894, in which the chief subjects under discussion were, first, what organisms were capable of producing hydrogen sulphide, second, from what materials the sulphur could be utilized by bacteria for the production of hydrogen sulphide, and third, whether aerobic or anaerobic conditions were most favorable for hydrogen sulphide production ; the evidence submitted and the opinions expressed by these workers being very diversified and often contradictory.

SCHARDINGER'S WORK.

Undoubtedly influenced by the large amount of work then being published on hydrogen sulphide production and by the discussions being waged at that time on the subject, Surgeon Major Dr. Fr. Schardinger[26], of Vienna, in 1894, saw the advantages of applying tests for hydrogen sulphide production to water examination, and it was he who originated the method, with an investigation of which the present paper has to deal.

Dr. Schardinger called attention to the fact that the bacteria causing typhoid fever, Asiatic cholera and inflammations of the stomach and intestines were ever present in the feces of infected persons and might hence find their way into sewage and subsequently into water supplies, and that with these organisms, *B. coli communis* is always associated in very large amounts. Therefore, he began looking about for a method to measure this "possibility of infection" as it had long before been termed by Dr. Hueppe.

The method which had previously been used, namely, ascertaining the number of germs in a fixed water mixture, as different kinds of organisms, he did not consider to be a good method, as the number found might be too much influenced by local conditions aside from the question of the "possibility of infection."

In searching about for the proper medium in which to cultivate the organisms which would show this "possibility of infection" he took into account the fact that Professor Nencki and his pupils had shown that in the small intestine of a well man, chiefly fermentative processes were carried on, whereas, in the large intestine, chiefly putrefactive processes were carried on ;

thus giving rise to two groups of bacteria, those causing fermentation and those causing putrefaction, "concerning which one must inform himself in a suspected water."

Giving credit to French investigators for first taking more than the customary one cubic centimeter of water for inoculation, Dr. Schardinger at first mixed seventy cubic centimeters of each of the samples under examination with thirty cubic centimeters of bouillon containing five percent of sugar, incubated at thirty seven degrees for twenty four hours and then plated this enriched culture with sugar gelatine or agar. The futility of this method is shown by his own admission that in many hundred examinations, he found *B. coli communis* only five times.

Later, besides the sugar bouillon, he used also a sterile solution of one gram of Witte peptone and one gram of sodium chloride in ten cubic centimeters of distilled water, which, mixed with one hundred cubic centimeters of the water to be examined, was kept for twenty-four hours at thirty seven degrees Centigrade. When this method was used, he says, "If the investigated water was impure, there appeared in a marked degree (a) a pronounced fecal-like odor (b) the turning yellow brown to black of a suspended paper strip whose surface was covered with a thin coating of lead carbonate, immediately or within a very short time afterward, due to the formation of lead sulphide, proving abundant formation of hydrogen sulphide."

Dr. Schardinger further stated that he obtained the fecal-like odor only from stool examinations or from fecal polluted waters.

He claimed as the result of later work that if less than fifteen milligrams of potassium nitrate was present per liter, that even waters of known purity would give hydrogen sulphide production. He further urged that for the application of the method, a known intensity of color on the lead carbonate paper within a definite time is necessary, for he says, "waters will give weak reactions also in which there is not the remotest possibility of infection existing, since almost every germ yields it."

More work followed by Karlpus [27], Beyerinck[28], [30], [31], [32], Zelinsky[29], and Orlowski[33] on the mechanism of the production of hydrogen sulphide by bacteria between the years of 1894 and 1896.

DUNHAM'S WORK.

In 1897, there appeared an article by E. K. Dunham[34] which seems to be the first contribution by an American to the subject of hydrogen sulphide production by bacteria. This article was on the value of a bacteriological examination of water from a sanitary point of view, and after pointing out that the methods then in use were insufficient, this author stated that "all sewage which receives human feces contains the bacillus coli communis, or if it does not, has been subjected to germicidal agencies that would also kill pathogenic bacteria, derived from the cases of disease. It is fair to assume that ordinary sewage would also contain the common bacteria of putrefaction. We must therefore direct our attention to the means of demonstrating the presence or absence of those species in the water under examination."

Dunham went on to say that he was inclined to believe that the best way of accomplishing this was by the application of the putrefactive test of Schardinger and gave the following as his method:—To about ninety cubic centimeters of water, ten cubic centimeters of a ten percent peptone, five percent salt solution, previously sterilized, were added. This gave a resulting solution containing one percent of peptone and five tenths percent of sodium chloride. The mixture was made in a sterile Erlenmeyer flask, provided with a cotton plug. A strip of paper, impregnated with lead carbonate, was suspended over the mixture and the flask was then placed in the incubator at thirty-seven degrees Centigrade for twenty-four hours. Under these conditions of temperature and nutrition, Dunham claimed that "the colon bacillus and the bacteria of putrefaction readily multiply and the latter cause the production of hydrogen sulphide which discolors the lead paper."

More work followed between 1897 and 1904 by Morris[35], Selensky and Brussilowsky[36], Beyerinck[37], Saltet[38], and von Delden[40], [41], chiefly on the questions of what bacteria could produce hydrogen sulphide; whether they were aerobes or anaerobes and whether they could utilize sulphates as sources of sulphur. The results of this work were well edited by Macé[42], in the fifth edition of his Traité Pratiqué de Bacteriologie which appeared in 1904 and

in which he stated that the bacteria of putrefaction were partly aerobes and partly anaerobes. He stated that in the process of putrefaction, hydrogen gas is given off which while nascent unites with sulphur to form hydrogen sulphide and with phoshorous to form phosphine, while at the same time, leucine, tyrosine, glycocoll and ptomaines are also formed.

Macé divided the process of putrefaction into three stages, in the first stage he stated that there were many ordinary saprophytes present such as *B. subtilis*, *B. mesentericus*, *B. vulgaris* and B. I, II and III described by Mouginet[43]; in the second stage, which appeared a day or two later, according to temperature, he stated that there was an increased odor, the ascendency being assumed by such organisms as *B. fluorescens liquifaciens*, *B. fluorescens putridus* and *B. violaceus;* in the third stage, which arrived several days later, he stated that there was a decidedly putrid odor, *Proteus vulgaris* and *Proteus mirabilis* predominating.

In 1904, Beyerinck[44], followed up his previous work with an article in which he laid down broad lines of oxidation and reduction, saying that the presence of free hydrogen was not always necessary to reduction. He said that it was necessary for anaerobes to have small amounts of free oxygen to start their development and that later they used the combined oxygen of organic substances and that therefore there were no true anaerobes in the strict sense of the word.

Work on the hydrogen sulphide producing ability of specific organisms and upon the materials which these organisms can utilize in their growth and development has been carried on between 1904 and 1912 by Rank[45], Huss[46], Hildebrandt[47], Belonowski[48], Paus[49], Nawiaski[50], Kestler[51], Porcher and Pamisset[52], Herter[53], Brasch[54], Dunschmann[55], McCrudden[56], Kendall[57], Weichardt[58], Lange and Pippe[59], Kuehel[60], Bainbridge[61] and Neuberg[62], but no further application of hydrogen sulphide production to water analysis has been made since the reference made to it by Dunham, so far as the author has been able to discover.

INTRODUCTION TO EXPERIMENTAL WORK.

The method for the detection of putrefactive organisms as proposed by Schardinger and modified by Dunham has been used for several years in the laboratory of Sanitary Chemistry at Cornell University and in the course of making many hundred examinations of water it was found to be of great value in forming an opinion as to the quality of the water, the great objection to it being that it was only with very badly polluted waters that positive results could be obtained in twenty four hours ; waters which were only slightly polluted requiring sometimes as long as ninety six hours incubation in order to give hydrogen sulphide production in the medium of either Schardinger or Dunham. Some preliminary work showed that the medium could be sensitized in various ways, and as the speed with which results can be obtained is a very vital point in the bacteriological examination of water, it seemed to the author that the method was worthy of a systematic study in order to ascertain the composition of the medium which would give positive results in the shortest length of time and with the greatest amount of uniformity and therefore, the present investigation was undertaken under the direction and kindly criticism of Professor E. M. Chamot.

CHAPTER II.

SPECIAL APPARATUS AND REAGENTS.

APPARATUS.

The only special form of apparatus which was used in the work and which needs mention was the form of flask in which the inoculated media were incubated. This flask is illustrated in Cut 1 at the end of the article. The flasks have been found to be well adapted to the purpose for which they were designed, the ground glass joint effectively preventing escape of gas and the tubular part of the cap being of such size that it forms a very convenient receptacle for a strip of bibulous paper which has been soaked in a ten percent solution of neutral lead acetate and dried. Before incubating, this tube was closed as a rule by winding a piece of tin foil closely around it, allowing the tin foil to project about ten millimeters above the top of the tube, and then twisting this hollow cylinder of tin foil into a tight twist. In all cases, no matter whether a tin foil cap was used or not, a loosely fitting plug of cotton wool was always inserted at the base of each tube before the flask with its contents was sterilized.

REAGENTS.

All of the salts, acids and alkalies used, were taken from the department's stock of C. P. chemicals.

As the source of organic C, H, O, N and S, Witte peptone was used throughout the work. Peptone from three different lots was used while the work was in progress and was found to run very uniform in quality as shown by the chemical and physical tests made upon it, which included :—

1. Determination of acidity, using phenol phthalein as indicator, at 20° C.

2. Determination of the specific gravity of solutions of different strengths at 20° C. by means of the Westphall balance.

3. Determinations of the coefficient of viscosity at 20° C. by means of the Dudley viscosity pipette.

These tests were all made on solutions prepared by boiling weighed amounts of the peptone in weighed amounts of water,

cooling, making up to the required weight and then filtering through paper. The results for all the three lots of peptone are shown graphically on Plates A, B and C at the end of the article.

SEWAGE SAMPLE USED FOR INOCULATIONS.

Whenever an artificial sewage was used for inoculating media, as was done throughout all of the first part of the work, it was prepared by adding .02 grams of fresh, moist feces to each liter of tap water employed. This was strained through a sterile cloth into a sterile beaker for use. It was found on making the appropriate tests that this sewage contained from 2,000 to 5,000 bacteria per cubic centimeter which would develope on standard gelatine plates incubated for 72 hours at 20° C., of which, from 100 to 200 developed on standard agar plates incubated for 72 hours at 38° C., and it was further found that from 20 to 40 bacteria of the B. coli group were present per cubic centimeter of the sewage.

The reason tap water was used in preparing the sewage was because it was considered advisable to provide conditions which would simulate as nearly as possible those in which the bacteria would normally exist, namely, a natural water supply with its small amounts of dissolved salts.

CHAPTER III.

EFFECT ON HYDROGEN SULPHIDE EVOLUTION PRODUCED BY FILTERING PEPTONE SOLUTIONS.

As is well known, what we ordinarily call a peptone solution is not a solution at all, but is a colloidal suspension, and when peptone is boiled with water there is always formed a heavy sediment which may properly be considered to be that part of the peptone that has not been ground fine enough to stay in suspension.

In order to ascertain whether this assumption was true and also in order to find out whether any effect would be produced upon hydrogen sulphide production if this undesirable sediment were filtered from the medium, the following tests were made :—

1. Quantitative determinations were made of the amounts of sulphur, nitrogen, ash and phosphates in the ash ; of the sediment and of the peptone ; and these various constituents were found to be practically identical in both, indicating very strongly that the material of which the sediment is composed is peptone. An attempt was made to grind the dried sediment fine enough so that it would go into colloidal suspension, but the attempt was only partially successful, probably due to the fact that we had no better grinding device available than a mortar and pestle. When so ground, a part of the powder would not pass through a 100 mesh sieve, showing very clearly that the grinding was not efficient.

2. In each of 15 of the special culture flasks illustrated in Cut I, was placed 10 cc. of unfiltered 30.0% peptone solution and .5 grams of sodium chloride. Another 15 flasks were similarly prepared except that filtered peptone solution was used in place of unfiltered. All of the flasks were plugged and sterilized, and then each was inoculated with 90 cc. of artificial sewage and incubated under the same conditions.

The amounts of hydrogen sulphide produced in the filtered and in the unfiltered media were practically identical, and as it is decidedly advantageous to start with a clear medium so as to detect the growth of bacteria by means of the turbidity and sedi-

ment produced in the medium ; from this point on, all peptone solutions were filtered before they were used.

SUMMARY.

All that part of peptone which is agglomerated on boiling with water is identical in composition with peptone, and as filtered and unfiltered peptone solutions show no difference in the amount of hydrogen sulphide produced in them, the use of clear, filtered solutions is much to be preferred.

CHAPTER IV.

EFFECT ON HYDROGEN SULPHIDE EVOLUTION OF DIFFERENT CONCENTRATIONS OF PEPTONE.

This part of the work was undertaken in order to ascertain whether some other concentration of peptone than the one gram in 100 cc. of inoculated medium as recommended by Schardinger[26] and later adopted by Dunham[34], or than the two grams per 100 cc. as had been used in the laboratory of Sanitary Chemistry at Cornell University, would furnish a medium in which the organisms producing hydrogen sulphide would perform this function more quickly and more energetically.

THE TRIANGULAR DIAGRAM.

As the character and amounts of the different inorganic salts which might be used to sensitize the medium and as the character and amounts of the different acids and bases which might be used in adjusting the reaction of the medium, were subjects which it seemed highly advisable to investigate at the same time that the concentration of the peptone was being investigated, the use of the triangular diagram on which three components can be plotted, was resorted to.

The diagram was used in the manner originally proposed by Gibbs and not in the manner later proposed by Roozeboom, which latter is the way in which it is now used by most physical chemists.

By using the diagram according to the Gibbs method, which it will be recalled, gave very excellent results in the hands of Oswald Schreiner of the Bureau of Soils, U. S. Dept. of Agric., in the study of plant nutrients, the concentrations of the three factors, the peptone, inorganic salts, and reaction adjustor could be varied in a systematic manner between wide limits, so as to find the maximum and minimum limits beyond which it would be useless to go in subsequent work, where only one component would be varied at a time. In other words, fields could be found within which the production of hydrogen sulphide would be most rapid and most energetic, and further work confined to those fields.

A word of explanation may facilitate the reading of the diagram by those unfamiliar with the Gibbs method. If reference be made to Plate I at the end of the article, it will be seen that, reading in a clock-wise direction, the peptone has been varied between 5.0% and 0.0% ; the reaction between 5.0% and 0.0% of normal acid ; and the inorganic salt (NaCl in this case) between 5.0% and 0.0%. All of the percents as indicated, are the percents as they exist after the media have been inoculated with sewage or water samples.

On the diagram, 66 different combinations are found at the intersections of the lines. Of these, the points numbered 1, 3, 6, 10, 15, 21, 28, 36, 45, 55 and 66 are all assigned values of 0.0% peptone ; the points numbered 2, 5, 9, 14, 20, 27, 35, 44, 54 and 65 are all assigned values of .5% peptone ; the points numbered 4, 8, 13, 19, 26, 34, 43, 53 and 64 are all assigned values of 1.0% peptone, etc. The points numbered 56, 46, 37, 29, 22, 16, 11, 7, 4, 2 and 1 are all assigned values of 0.0% NaCl ; the points numbered 57, 47, 38, 30, 23, 17, 12, 8, 5 and 3 are all assigned values of .5% NaCl ; the points numbered 58, 48, 39, 31, 24, 18, 13, 9 and 6 are all assigned values of 1.0% NaCl, etc. The points numbered 66, 65, 64, 63, 62, 61, 60, 59, 58, 57 and 56 are all assigned values of 0.0% normal acid ; the points numbered 55, 54, 53, 52, 51, 50, 49, 48, 47 and 46 are all assigned values of .5% normal acid ; the points numbered 45, 44, 43, 42, 41, 40, 39, 38 and 37 are all assigned values of 1.0% normal acid, etc. To further illustrate and explain, the composition of the medium at several different points chosen at random will be as follows :—

Point 1,	0.0% peptone,	0.0% NaCl,	5.0% normal acid.
5,	.5	.5	4.0
13,	1.0	1.0	3.0
17,	2.0	.5	2.5
30,	3.0	.5	1.5
50,	2.5	2.0	.5
57,	4.5	.5	0.0

It will be noted that with the diagram as applied in Plate I, only low concentrations of NaCl and of acidity are given with high concentrations of peptone ; that with high concentrations of NaCl only low concentrations of peptone and of acidity are given ; and with high concentrations of acidity, only low con-

centrations of NaCl and of peptone are given. As we desired combinations in which salts and acidity would be high when peptone was low, and also combinations in which one would be high when the other was low, as well as having either high or low when the peptone was high, recourse was had to a series of diagrams in which first the order of concentration of one component was reversed and then another. A series of eight diagrams covers all of the possible combinations and Plates II, III and IV at the end of the article illustrate such modified diagrams.

RESULTS WITH PEPTONE AND SODIUM CHLORIDE.

Flasks of media were prepared with compositions as shown by the different points on the diagrams as given in Plates I, II, III and IV. These were inoculated with equal amounts of sewage and incubated at 38° C. (blood temperature), and observed at frequent intervals for the first appearance of the blackening of the strips of paper impregnated with lead acetate which had been placed in the tubular top of the caps of the flasks.

It was found in these four runs that the respective fields in which hydrogen sulphide was most quickly developed were as follows :—

3.0 to 4.5% peptone ; 0.0 to 1.5% NaCl ; 0.0 to 2.0% N. acid.
2.5 -- 5.0 ---------- 1.0 -- 3.0 -------- 1.0 -- 2.5
4.0 -- 5.0 ---------- 0.0 -- 2.5 -------- 0.0 -- 2.5
4.5 -- 5.0 ---------- 2.0 -- 2.5 -------- 2.5 -- 3.0

From a careful study of all of the data obtained in the four runs, it was found that the very best conditions had been obtained within the following limits :—

2.5 to 5.0% peptone ; 0.5 to 1.5% NaCl ; 0.5 to 1.5% N. acid.

TWO COMPONENT DIAGRAMS.

Consequently, a run was now made, the results of which are given in Plate V at the end of the article, in which all of the possible combinations of 2.0%, 2.5%, 3.0%, 3.5% and 4.0% of peptone ; 0.5%, 1.0% and 1.5% of NaCl ; and 0.5%, 1.0% and 1.5% of normal acid were used, plotted on two component diagrams.

Four checks with 2.0%, 2.5%, 3.0% and 4.0% of peptone, with no added salt and with a reaction of 1.0% normal acid were also prepared.

It was found in the uninoculated media, that of the combinations prepared, the only ones which were clear, with very little sediment, were those containing 3.0% of peptone, 1.0% and 1.5% of NaCl, and with an acidity to phenol phthalein of 1.5% normal acid. It was moreover, a general rule that the amount of peptone and of sodium chloride did not seem to have much influence on the amount of turbidity in the solution, but that the reaction did have such an influence; the less the acidity, the greater the turbidity; but there were many exceptions.

Each flask was inoculated with 90 cc. of artificial sewage.

As regards the turbidity of the inoculated media before incubation, none were perfectly clear, with no sediment, but the best combinations were all of those in which the reaction was equivalent to 0.5% normal acid, and where NaOH had been used to adjust the reaction.

The inoculated flasks were incubated at 38°C. and frequently observed for evidences of hydrogen sulphide production.

As regards the rapidity of hydrogen sulphide production, it was found to be most rapid in those combinations containing 3.5% and 4.0% of peptone, the concentration of the sodium chloride and the reaction of the media apparently not making a great deal of difference, in the narrow limits used.

The largest amounts of hydrogen sulphide were produced in general in the flasks having from 3.0% to 4.0% of peptone; from 0.5% to 1.5% of NaCl; and with a reaction equivalent to from 1.0% to 1.5% normal acid. These were also the combinations in which the media became the most turbid after incubation and in which the largest amounts of grayish green sediment were produced. It was also noted that in most of these flasks, there were particles of dark green material floating on the surface which sank on tapping the flasks.

In order to obtain more reliable information as to which combinations were really best and producing hydrogen sulphide most rapidly, it was deemed advisable to make up in triplicate those combinations which had given the best results in the run as illustrated on Plate V. The compositions of the media and the results are presented in Plates VI and VII at the end of the article. While none of the combinations used were perfectly clear and free from sediment either before or after inoculation,

they were near enough so for all practical purposes so far as judging how far growth and development of the bacteria had proceeded on incubation.

The results of all of the combinations so far cited showed that when sodium chloride was the inorganic salt present, that there was not much choice between 3.0%, 3.5% and 4.0% of peptone, all of which were much superior to 2.0% and 2.5% of peptone.

PEPTONE IN PRESENCE OF SALTS OTHER THAN SODIUM CHLORIDE

Runs were made similar in all respects to those the results of which are shown in Plates I, II, III, IV, V, VI and VII; in which the sulphates, nitrates and phosphates of sodium were substituted for sodium chloride; and also in which the chlorides, sulphates, nitrates and phosphates of potassium, ammonium, calcium and magnesium were substituted for sodium chloride and in which the reaction was adjusted in a variety of ways.

Without giving the details of the results of each run, suffice it to say that they all bore witness to the same fact, namely, that from 3.0% to 4.0% of peptone furnished the best concentration for most rapid and most energetic production of hydrogen sulphide by the bacterial floras employed.

EFFECT OF THE ADDITION OF BEEF EXTRACT TO THE MEDIUM.

A run was made in which beef extract was added to the medium to see what effect, if any, would be produced on hydrogen sulphide evolution.

The beef extract was made up by digesting 2100 grams of chopped beef with 4200 grams of tap water for one hour at 60°C. The digested material was then strained through cheese cloth, the last part of the liquid being pressed out with a strong iron press. The liquid was then boiled for twenty minutes, cooled, the fat skimmed off, and made up to 4200 cc. with tap water. Normal NaOH solution was then added until the liquid was neutral to phenol phthalein at 20°C. It was then boiled for forty-five minutes and filtered while hot through paper. The extract was found to contain 2.216% of solids and gave .686% of ash. It was perfectly clear both before and after sterilization.

Five flasks of each of ten different combinations of beef extract, peptone and potassium chloride, as shown in Table I were prepared.

TABLE I.

Flask numbers	cc. beef extract	cc. 30% peptone	% added peptone.	grams KCl added	cc. water added
1 to 5	0.	10.	3.00	.5	55
6 to 10	0.	10.	3.00	0	55.
11 to 15	50.	10.	3.00	.5	5.
16 to 20	50.	10.	3.00	0	5.
21 to 25	50.	7.5	2.25	.5	7.5
26 to 30	50.	7.5	2.25	0	7.5
31 to 35	50.	5.	1.50	.5	10.
36 to 40	50.	5.	1.50	0	10.
41 to 45	50.	0.	.00	.5	15.
46 to 50	50.	0.	.00	0	15.

When the components of the above combinations were added together, it was found that flasks 1 to 5 and 41 to 50 were perfectly clear, whereas, all of the others were turbid. Flasks 6 to 10 contained no sediment but 11 to 40 contained a faintly yellow sediment which decreased with decreasing concentration of peptone.

From the work which was done in connection with phosphates (to be mentioned later) and from the appearance of the sediment, it is justifiable to assume that the sediment consisted of peptone agglomerated by the phosphate which is always present in meat extract. This sediment was not filtered out.

After inoculation, the condition of the media was not changed from that noted above. As each flask contained 65 cc. of medium before inoculation, 35 cc. of the artificial sewage was added to each, making the final volume 100 cc.

After 18 hours of incubation, hydrogen sulphide began to appear in many of the flasks; numbers 1 to 10, 35 to 43, and 49 being the only flasks not then showing hydrogen sulphide. Those flasks to which 2.25 grams of peptone had been added to 50 cc. of the beef extract gave the most rapid production of hydrogen sulphide.

That practically all of the flasks in which the beef extract was present gave hydrogen sulphide in 18 hours, while none of the flasks containing the 3.0% peptone, .5% potassium chloride, mixture, gave hydrogen sulphide in this length of time shows

that there is something contained in the beef extract which is needed by the putrefactive organisms for most rapid production of hydrogen sulphide and which is not present in peptone.

After 24 hours of incubation, the bacteria in the 3.0% peptone, .5% KCl medium had begun to produce hydrogen sulphide, but the bacteria in the media containing beef extract had produced much larger amounts. However, the additional labor involved in the use of beef extract does not seem to be warranted, especially in view of the fact that its composition and nutritive value are admitted to be different in every lot prepared. This circumstance would tend to alter the length of time required for a definite amount of pollution with putrefactive organisms to show hydrogen sulphide production and hence would obviate the possibility of using the length of time for hydrogen sulphide production as a rough measure of the amount of sewage pollution.

The presence of potassium chloride in the media containing beef extract proved to be a distinct aid in the production of hydrogen sulphide.

One undesirable feature was that the media in all of the flasks containing beef extract remained yellow in color even after much hydrogen sulphide had been produced, and what little sediment there was present was light yellow in color, while the 3.0% peptone, .5% potassium chloride media all turned turbid and green with dark green sediment and dark green floating cakes.

The flasks containing 3.0% peptone without potassium chloride showed much less hydrogen sulphide produced than was the case when potassium chloride was present.

SUMMARY.

1. Irrespective of the inorganic salts present and of the acidity of the medium, a concentration of from 3.0% to 4.0% of peptone in the inoculated medium is the best for the most rapid and most energetic production of hydrogen sulphide by putrefactive bacteria. It is recommended that 3.0% of peptone be used as being more economical and also easier to handle than higher concentrations.

2. Although the addition of beef extract to peptone materially reduced the length of time required for hydrogen sulphide production, the additional labor involved does not appear to be warranted, especially in view of its varying composition.

CHAPTER V.

EFFECT ON HYDROGEN SULPHIDE EVOLUTION PRODUCED BY DIFFERENT INORGANIC SALTS.

From the data obtained in the experiments, the results of which are given in Plates I, II, III, IV, V, VI and VII, it was very strongly indicated that the presence of NaCl in the peptone medium was of benefit in obtaining the most rapid and most energetic production of hydrogen sulphide, together with the other manifestations of the growth and development of putrefactive organisms, such as the greening of the solution and the production of turbidity therein ; and the formation of dark green or grayish green sediment in the media and of dark green floating cakes thereon.

This conclusion was reached by a comparison of the results obtained with those media which had received sodium chloride and those media to which no sodium chloride had been added, the media to which sodium chloride had been added showing a decided superiority on such a comparison. The question which naturally presented itself was, "Is sodium chloride the best salt to use for sensitizing the medium or would some other salt be even better?" To gain information on this point, a series of runs was now made in which sodium sulphate, sodium nitrate, potassium chloride, magnesium chloride, calcium chloride and ammonium chloride were respectively substituted for the sodium chloride.

MODIFIED USE OF TRIANGULAR DIAGRAM.

Advantage was taken of the experience which had been gained in making the runs with sodium chloride, so that with a simple change in the arrangement of the diagram, it was possible to gain as much information from the use of one triangular diagram for each of these salts as had been obtained from running four separate diagrams with sodium chloride. The modified diagram read in a clock-wise direction in the following manner :—1.0 to 6.0% peptone ; 5.0 to 0.0% N. acid ; 5.0 to 0.0% salt.

Starting with 1.0% peptone instead of 0.0% had the following advantages :—it eliminated the 0.0% and .5% peptone solutions,

which had been found in the previous work to be of no use ; and it placed the 2.5%, 3.0% and 3.5% peptone solutions in that part of the diagram where the concentrations of salt and acidity had been found to be the best suited to good growth and hydrogen sulphide production.

In the previous runs, peptone solutions of the different concentrations desired, had been made up separately, but from this point on, only 30.0% peptone solution was prepared, of which proportional amounts were used to give the desired concentrations ; for example, 6.7 cc. was used for a 2.0% solution, 10.0 cc. for a 3.0% solution, 13.3 cc. for a 4.0% solution, etc., the difference between the amount taken and 15 cc. being made up with boiled, cooled and filtered tap water. (The reason why tap water was used in this case as in the case of making all of the media employed, was so as to furnish to the bacteria an environment as nearly as possible identical to the one in which they would normally exist, namely a natural water supply with its small amounts of dissolved salts). This had the advantage of using the same identical solution of peptone for all of the inoculations and also of making the uninoculated media 50.0% larger in volume, thereby better keeping all of the ingredients in solution.

The results of the runs with sodium chloride and with potassium chloride, which salts proved to have the most beneficial effect, are given in Plates VIII and XI at the end of the article. In Plate XV, for ease of comparison, the limits or fields within which the best results were obtained with potassium chloride, sodium chloride, sodium sulphate, calcium chloride, magnesium chloride and ammonium chloride, have been plotted as a composite diagram.

CHECK SOLUTIONS USED.

In all of these runs, the same check solutions were used in order to make the runs as nearly comparable as is possible when a fresh lot of sewage must be prepared for each run. These check solutions had the following compositions :—

No. 30a	3.0% peptone ;	0.5% NaCl ;	1.5% N. acid.	
31a	3.5	1.0	1.5	
32a	4.0	1.5	1.5	
56a	3.0	0.5	1.02	(unadjusted)

The check solutions were all practically clear both before and after inoculation and in all of the runs they gave results so nearly alike that these runs may be considered as entirely comparable.

Without giving in detail the results of each of these runs, the following deductions are presented.

RESULTS WITH SODIUM CHLORIDE.

The best results were obtained with from 0.5 to 1.5% of sodium chloride, more than 1.5% inhibiting the growth and activity of the putrefactive organisms. The sodium chloride is beneficial in keeping the peptone from agglomerating.

RESULTS WITH SODIUM SULPHATE.

The sewage used in this run was somewhat more active than that used in the other runs of this series, as shown by the check flasks, but notwithstanding that fact, the sodium sulphate did not have any more beneficial effect than did the sodium chloride. The best results were obtained with from 0.5% to 1.5% of sodium sulphate, the presence of more than 1.5% tending to decrease the amount of hydrogen sulphide formed. Sodium sulphate in small amounts is beneficial in keeping the peptone from agglomerating.

RESULTS WITH POTASSIUM CHLORIDE.

As much as 3.0% of this salt may be present without inhibiting the activity of the bacteria and without interfering with the evolution of hydrogen sulphide.

In concentrations less than 3.0% and especially in a concentration of about .75%, the potassium chloride has a very beneficial influence on hydrogen sulphide production.

This run furnished the first indication that potassium chloride was superior to any other salt for sensitizing the medium, an indication which was to be verified many times in subsequent work.

It should be noted that as the concentrations of peptone and of acidity decreased in this run, the amount of potassium chloride necessary increased, probably due to the increasing need of an ionizing salt to make the decreasing amounts of peptone avail-

able as food for the bacteria. This explanation is based upon the belief that the presence of ionizing salts aids in the hydrolysis of the complex peptone into simpler amino compounds, which may be used by the bacteria more readily as nutritive material.

Potassium chloride helps to keep the peptone from agglomerating.

RESULTS WITH MAGNESIUM CHLORIDE.

This salt has slight beneficial influence in concentrations of from 0.5% to 1.0%. More than 2.0% has an inhibiting influence.

RESULTS WITH CALCIUM CHLORIDE.

Calcium chloride seemed to be about equal to magnesium chloride, except that its field was somewhat differently located. It did not exert an inhibiting action until more than 2.0% was present, but in this case an acidity equivalent to 1.5% was required. When there was more than 2.5% of acidity, the calcium chloride did not have any beneficial influence, thus showing the most restricted field of any of the inorganic salts so far considered, namely, from 0.5% to 2.0% of the salt, with from 1.5% to 2.5% normal acid.

RESULTS WITH AMMONIUM CHLORIDE.

This always had a beneficial influence in concentrations of from 0.0% to 1.5% with acidities varying from 0.0% to 3.0% but by no means did it have such beneficial influence over all of this field, as shown in the composite diagram, its best influence being shown at the lower concentrations of acidity. This is believed to be due to both the beneficial influence of its ionization and also to the probable condition that its nitrogen was to some extent used as food, as the concentration of the peptone was also low when the acidity was low in the field in which the ammonium chloride had a beneficial influence.

RESULTS WITH SODIUM CHLORIDE, POTASSIUM CHLORIDE, AMMONIUM CHLORIDE, CALCIUM CHLORIDE, MAGNESIUM CHLORIDE, SODIUM SULPHATE, POTASSIUM SULPHATE, AMMONIUM SULPHATE, CALCIUM SULPHATE, MAGNESIUM SULPHATE, SODIUM NITRATE, POTASSIUM NITRATE, AMMONIUM NITRATE, CALCIUM NITRATE AND MAGNESIUM NITRATE.

From the results as given in Plates VIII, XI and XV, there was a strong indication that potassium was the best basic ion to have present in the inorganic salt added to sensitize the medium and that chlorine was the best acid ion to have present for this same purpose, but it was felt that more evidence was essential before a definite statement could be made and especially did it seem necessary to try as many different combinations of basic and acid ions under exactly the same conditions as possible. In order to use as many such combinations as possible and still keep the number of inoculations within what had been found could be handled at one time it seemed best to keep the concentrations of peptone and of acidity constant and to vary the composition and concentration of the inorganic salts only. A concentration of 3.0% was chosen for the peptone as that had been found to work as well as any in the inoculations, numbering about eight hundred, which had been made in the investigation so far. As to the concentration of acidity, it had been noted that the check flasks used in the runs represented in Plates VIII to XIV had always given as good results as any of the other combinations, and as it had been further noted that No. 56a check flask almost invariably gave the best results among these checks, and as No. 56a was in all cases made up with reaction unadjusted ; and as it had been found in these same eight hundred inoculations mentioned in connection with peptone above, that from 1.0% to 1.5% of normal acid furnished more favorable conditions than any other reaction, and as the acidity of an unadjusted 3.0% peptone solution had been found to be equivalent to 1.02% of normal acid ; and as, moreover, it had been found as the result of sixteen inoculations that practically identical results were obtained when an unadjusted medium was used as when the medium was first made neutral to phenol phthalein with a fixed

alkali and then, with hydrocloric acid, brought to the same degree of acidity as the unadjusted medium; the 3.0% peptone was left unadjusted with a reaction equivalent to 1.02% normal acid.

The salts which were used were those mentioned in the heading of this section. Two check flasks were also prepared containing no salt. These were all inoculated from the same lot of sewage and incubated under identical conditions.

The results of this set of experiments are given in Table II and from these results it is seen that the quickest and most abundant evolution of hydrogen sulphide was obtained in those flasks containing 0.5% potassium chloride, 1.0% potassium chloride, 0.5% sodium chloride, 1.0% sodium chloride, 0.5% potassium sulphate, 0.5% calcium sulphate, 1.0% magnesium sulphate and 1.5% magnesium sulphate; the results with potassium chloride being considerably better than with the other salts mentioned, although all of these salts had a beneficial influence as shown by the fact that hydrogen sulphide production was more rapid in their presence than it was in the check flasks to which no salt had been added.

That nitrates had an inhibiting influence is in accord with the theory that nitrates have an oxidizing effect, while the production of hydrogen sulphide is essentially a reduction process.

TABLE II.

Salt present.	% salt.	Millimeters blackened of lead acetate paper in— 24 hours.	48 hours.	72 hours.
None	0	0	12	all
	0	0	14	all
NaCl	.5	2	all	all
	1.0	4	all	all
	1.5	3	all	all
KCl	.5	13	all	all
	1.0	16	all	all
	1.5	9	all	all
NH_4Cl	.5	2	all	all
	1.0	0	all	all
	1.5	0	all	all
$CaCl_2$	.5	5	all	all
	1.0	0	all	all
	1.5	0	all	all

TABLE II.—Continued.

Salt present.	% salt.	Millimeters blackened of lead acetate paper in— 24 hours.	48 hours.	72 hours.
$MgCl_2$	.5	0	all	all
	1.0	0	all	all
	1.5	0	all	all
Na_2SO_4	.5	0	all	all
	1.0	0	all	all
	1.5	0	all	all
K_2SO_4	.5	16	all	all
	1.0	5	all	all
	1.5	3	all	all
$(NH_4)_2SO_4$	.5	0	all	all
	1.0	0	all	all
	1.5	5	9	15
$CaSO_4$	.5	13	all	all
	1.0	3	all	all
	1.5	0	all	all
$MgSO_4$	.5	0	all	all
	1.0	8	all	all
	1.5	11	all	all
$NaNO_3$	.5	0	3	10
	1.0	0	10	all
	1.5	0	3	7
KNO_3	.5	2	10	all
	1.0	0	13	all
	1.5	0	11	all
NH_4NO_3	.5	0	9	all
	1.0	0	8	all
	1.5	1	2	2
$Ca(NO_3)_2$	.5	1	6	9
	1.0	2	9	all
	1.5	0	7	9
$Mg(NO_3)_2$	.5	0	5	7
	1.0	0	4	4
	1.5	0	0	1

It seemed advisable to now make another set of experiments in which the five salts which had been found to give the best results should be varied within narrower limits and in which each concentration should be prepared in duplicate. All were prepared with 3.0% peptone, unadjusted. Five flasks containing 3.0% peptone with no added salt were prepared as checks. All of the flasks were inoculated from the same lot of sewage and were incubated under identical conditions.

The results of this set of experiments are given in Table III and from these results it is evident that potassium chloride in concentrations of from 0.5% to .75% induced the most rapid and the most energetic production of hydrogen sulphide.

TABLE III.

Salt present.	% salt.	Millimeters blackened of lead acetate paper in 18 hrs.	24 hrs.	30 hrs.	36 hrs.
None	0	0	0	4	13
	0	0	1	3	15
	0	0	2	3	16
	0	0	0	1	9
	0	0	0	4	16
NaCl	.25	0	3	8	18
	.25	0	4	10	18
	.50	0	4	12	all
	.50	0	3	14	all
	.75	0	4	16	all
	.75	0	5	15	all
	1.00	0	2	6	15
	1.00	0	1	3	12
KCl	.25	0	12	18	all
	.25	0	9	16	all
	.50	2	13	all	all
	.50	4	15	all	all
	.75	3	14	all	all
	.75	5	18	all	all
	1.00	4	11	18	all
	1.00	2	10	17	all
K_2SO_4	.25	0	3	13	16
	.25	0	5	14	all
	.50	0	6	all	all
	.50	0	3	12	18
	.75	0	2	12	all
	.75	0	6	18	all
	1.00	0	4	16	all
	1.00	0	3	10	17
$CaSO_4$	.25	0	3	14	18
	.25	0	0	7	15
	.50	1	7	17	all
	.50	0	4	12	all
	.75	1	5	12	all
	.75	0	3	10	18
	1.00	0	4	13	18
	1.00	0	2	10	16

TABLE III.—Continued.

Salt present	% salt	Millimeters blackened of lead acetate paper in 18 hrs.	24 hrs.	30 hrs.	36 hrs.
$MgSO_4$	.25	0	0	7	12
	.50	0	0	5	9
	.75	0	2	8	13
	.75	0	0	5	12
	1.00	0	2	8	15
	1.00	0	3	9	18
	1.25	0	3	11	18
	1.25	1	4	11	all
	1.50	0	6	15	all
	1.50	0	2	9	16
Na_2SO_4	.25	0	0	7	14
	.50	0	1	9	15
	.75	0	2	8	12
	1.00	0	0	4	10

From the results shown in Tables II and III, it is obvious that potassium is the best basic ion to have present ; that with the alkalies, chlorine is the best acid ion, and that with the alkaline earths, sulphur tetroxide is the best acid ion.

RESULTS WITH PRIMARY PHOSPHATE.

As phosphates have been so much used in preparing all sorts of media and as the statement has been so often repeated that their presence is absolutely essential to the best conditions for the life and activity of bacteria and for the activity of enzymes, the study of the effect of the presence of phosphates was taken up with high hopes of greatly augmented hydrogen sulphide production in their presence. As primary phosphate was the form recommended where an acid medium was desired, primary potassium phosphate was the first salt tried, using concentrations of the phosphate varying from 0.0% to 1.5% in a 3.0% peptone which was left unadjusted. Checks were prepared containing 0.5% of potassium chloride, 0.5% of sodium chloride and 0.5% of potassium sulphate in a 3.0% unadjusted peptone solution.

All of the solutions containing primary potassium phosphate precipitated badly when sterilized, those containing the least amounts giving only a small amount of precipitate while those containing the largest amounts produced what was practically a turbid jelly. This was due to the fact, as has been pointed out by colloidal chemistry, that the triply charged PO_4 ion has a

marked tendency to cause the colloids, of which peptone is one, to agglomerate. By adding a sufficient amount of strong hydrochloric acid, this turbidity can all be removed but the resulting media would be so acid that the growth and development of the bacteria would be entirely inhibited.

The practice is sometimes resorted to of filtering media to which phosphates have been added, leaving a filtrate of absolutely unknown strength as far as the concentrations of peptone and of inorganic salts are concerned, which resulting composition will depend on the temperature, reaction of the media and the initial concentrations of the different ingredients of the media. As this seems to be a very unscientific and therefore unwise procedure, such filtration was not resorted to in the present case.

As the result of this run, it was found that those check flasks which contained potassium chloride gave the most rapid and abundant production of hydrogen sulphide, followed in order by those containing sodium chloride and by one flask out of fifteen which contained no added salt. Out of twenty-four flasks to which primary potassium phosphate, in any amount, had been added, none showed any production of hydrogen sulphide in 24 hours and even after 72 hours of incubation, only thirteen of them showed even traces of hydrogen sulphide, while all of the fifteen flasks which had received no salt, had produced large amounts of hydrogen sulphide in 72 hours, showing that the influence of the phosphate was decidedly inhibiting.

RESULTS WITH SECONDARY PHOSPHATES.

As it had been noted that an initial acidity of from 1.0% to 1.5% furnished the best conditions for hydrogen sulphide production by bacteria and as the acidity was found by titrations to be increased to approximately 5.0% when as small an amount of primary phosphate as 0.5% was present, attention was now turned to secondary phosphates which are nearly neutral to phenol phthalein.

The secondary phosphates that were employed were those of calcium, magnesium, sodium, potassium and ammonium; and the mixed secondary phosphates of potassium and sodium, potassium and ammonium, and sodium and ammonium. These were introduced into a 3.0% unadjusted peptone solution in such

amounts as to give .05%, .15% and .25% of the basic element or elements of the various phosphates. As checks, flasks were prepared in duplicate with potassium chloride, sodium chloride, and potassium sulphate, introduced in such quantities as to furnish 0.05%, 0.15% and 0.25% of the basic element; also two flasks were prepared containing no added salt.

A heavy precipitate was formed in the flasks containing secondary phosphates, as had been the case with primary phosphates, the amount of precipitate increasing with increase of phosphate.

Moreover, it was found that during sterilization, considerable amounts of hydrogen sulphide were produced in the phosphate-containing media by purely chemical reaction. It was proved that the first observations were correct in this regard by repeating the phenomenon with flasks and plugs which had never been used before. Most hydrogen sulphide was produced chemically in the presence of ammonium and least in the presence of magnesium.

The results of the run showed that secondary magnesium phosphate gave the quickest and the best results of any of the secondary phosphates, followed in the order named by mixed potassium-sodium, secondary potassium, mixed potassium-ammonium, secondary sodium, secondary calcium, mixed sodium-ammonium and secondary ammonium phosphates.

None of the solutions containing secondary phosphates gave as good results as any of the check flasks, even those containing no added salt; showing that the presence of secondary phosphates in the concentrations employed was of no benefit in the production of hydrogen sulphide by the bacteria of the flora with which the inoculations were made.

In this run, for quickest production of hydrogen sulphide, for uniformity of results in duplicate flasks and for the production of a turbidity and green color in the inoculated media with production of dark green sediment and dark green floating cakes of material, potassium chloride once more showed itself superior to either sodium chloride or potassium sulphate.

It should be especially noted that of all of the flasks containing secondary phosphate, forty-eight in number, in this run, there was only one, containing 0.05% of calcium, which showed any

greening of the solution or production of dark green sediment or cakes even after 72 hours of incubation, and the duplicate of this flask did not show any of these features at all.

RESULTS WITH TERTIARY PHOSPHATES.

For the sake of completeness, a run was made in which tertiary phosphates were used, although it was a foregone conclusion that these would give even poorer results than were obtained with the primary and secondary phosphates because of the fact that the solutions made up with tertiary phosphates would be alkaline to phenol phthalein.

Tertiary magnesium, tertiary calcium, mixed magnesium-ammonium, tertiary ammonium, tertiary sodium, tertiary potassium, mixed sodium-potassium and mixed potassium-sodium phosphates were used in such amounts as to furnish 0.05% of the basic element or elements. Nine flasks were prepared containing each of the phosphates. As checks, flasks containing potassium chloride, sodium chloride and potassium sulphate in such quantities as to give 0.25% of the basic element were prepared in triplicate. Also three flasks containing 0.5% of potassium chloride, equivalent to 0.26% of potassium were prepared. These last three flasks were prepared so as to give a basis of comparison between the earlier runs, in which 0.5% of potassium chloride, and of sodium chloride, and of potassium sulphate had been used, and the later runs, in which sufficient inorganic salt had been employed to furnish 0.25% of basic elements or element.

As a result of the run, it was found that only traces of hydrogen sulphide were formed in the flasks which had received tertiary phosphates while all of the check flasks showed large amounts of hydrogen sulphide formed in 24 hours. In this particular run, one of the variations from the normal which are to be expected at times when one is dealing with a mixed flora, occurred, and more rapid evolution of hydrogen sulphide was obtained in the presence of sodium chloride than in the presence of potassium chloride, but the results with potassium chloride were more uniform than were those with sodium chloride, which fact would outweigh the benefits of the formation of hydrogen sulphide more rapidly in some of the solutions which contained sodium chloride.

RESULTS WITH VARIOUS COMBINATIONS OF POTASSIUM CHLORIDE, SODIUM CHLORIDE AND POTASSIUM SULPHATE IN CONJUNCTION WITH SECONDARY POTASSIUM AND SECONDARY SODIUM PHOSPHATES.

In order to cover every possible feature of the case with phosphates, it seemed best to use the secondary phosphates which had given the best results when used alone, in conjunction with potassium chloride, sodium chloride and potassium sulphate.

The following combinations were used in conjunction with a 3.0% unadjusted peptone solution :—

.200 grams K from KCl with	.05 grams K from K_2HPO_4
.150	.10
.175	.05
.100	.10
.200	.05 gram Na from Na_2HPO_4
.150	.10
.200 grams Na from NaCl with	.05 grams K from K_2HPO_4
.150	.10
.200	.05 grams Na from Na_2HPO_4
.150	.10
.200 grams K from K_2SO_4 with	.05 grams K from K_2HPO_4
.150	.10
.200	.05 grams Na from Na_2HPO_4
.150	.10

As checks, flasks were prepared containing in 3.0% unadjusted peptone solution, .25% of basic element furnished by potassium chloride, sodium chloride and potassium sulphate respectively and made up in triplicate.

After sterilization and before inoculation, all of the flasks containing phosphate, even in the presence of chlorides and sulphate, had very appreciable amounts of sediment in them. The same conditions held in the flasks after inoculation, with the difference that even more sediment was present, due probably to the formation of some tertiary calcium phosphate from the calcium salts in the artificial sewage.

As a result of the run, it was found that only about one-half of the flasks containing phosphate showed any hydrogen sulphide in the time required for the check flasks to develope large amounts, the first four combinations in the foregoing list showing small amounts. In none of the flasks containing phosphate had the solution turned green in color or shown dark green

sediment or floating cakes. Of the checks, those containing potassium chloride gave quickest and most consistent results.

As regards the effect of phosphates in general, it may be stated that so far as hydrogen sulphide production is concerned, either they are of no advantage or else the very small amount which is normally present in drinking waters, averaging somewhere in the neighborhood of 0.0001% as PO_4, is all that is required and that consequently the addition of more has an inhibiting influence. This explanation is founded on the theory of Huene[97], that bacterial growth is stimulated by very small amounts of bacterial poisons.

RESULTS WITH CALCIUM CARBONATE.

It had been noted in those flasks in which much hydrogen sulphide was produced, that the acidity to phenol phthalein approached 3.0%. The natural assumption was that this increased acidity came from the hydrolysing of the peptone into simpler amino acids which would increase the acidity, and possibly from the production of acetic, lactic acids, etc., and the question naturally arose "Is it not possible that if some carbonate, which itself will not effect the reaction of the medium but which will tend to neutralize the acid products of bacterial activity; is introduced, that by so neutralizing these byproducts it may materially aid the bacteria in producing hydrogen sulphide?" Calcium carbonate was first tried and was introduced in a concentration of 0.5% in combination with the following:—

.25% K from KCl.
.25% Na from NaCl.
.25% K from K_2SO_4.
.20% K from KCl and .05% K from K_2HPO_4.
.15% K from KCl and .10% K from K_2HPO_4.
.175% K from KCl and .075% K from K_3PO_4.
.10% K from KCl and .150% K from K_3PO_4.
.225% K from KCl and .025% K from KH_2PO_4.
.20% K from KCl and .05% Na from Na_2HPO_4.
.15% K from KCl and .10% Na from Na_2HPO_4.

These were all made up in triplicate and as checks, the same combinations without calcium carbonate were made up in triplicate. All were inoculated with artificial sewage.

There was a heavy sediment of insoluble calcium carbonate present throughout the run which is an undesirable feature as it

interferes with the forming of an opinion, by means of the presence in the culture of sediment and turbidity produced by the bacteria, as to whether or not the organisms are developing.

As a result of the run, it was found that hydrogen sulphide was developed rapidly and in large amounts in just about as many flasks that contained no carbonate as was the case when the carbonate was present, leading to the conclusion that calcium carbonate has no beneficial influence on the production of hydrogen sulphide by bacteria. Moreover, it is objectionable because of the sediment which it gives in the cultures.

RESULTS WITH PRIMARY SODIUM CARBONATE.

It was deemed advisable to try a soluble carbonate and as it was desired to use one which would not influence the reaction of the medium to phenol phthalein, primary sodium carbonate was chosen. The carbonate, in amount sufficient to give a concentration of 0.25% in the final inoculated medium, was used in combination with potassium chloride, sodium chloride and potassium chloride respectively, each of these salts being used in amounts to give 0.25% of the basic element present. As controls, flasks were prepared in triplicate containing 0.25% primary sodium carbonate without other salt ; and with 0.25% of basic element furnished by potassium chloride, sodium chloride and by potassium sulphate without any primary sodium carbonate being present ; and also three flasks containing no added salt.

As the result of the run, it was found that after sterilization and before inoculation, all of the flasks containing the added primary carbonate showed an undesirable, slight turbidity, and a marked deposit in the form of an adherant white film on the glass. This deposit, after thorough washing, was tested by microchemical means and proved to be calcium carbonate in the form of sphero-crystals of minute size. After the flasks had been inoculated and incubated, this film still persisted, being slightly augmented. A quantitative determination of the amount of calcium carbonate was made by titrating hot with tenth normal hydrochloric acid, using ethyl orange as the indicator and it was found to be equivalent to 0.33%.

As far as the production of hydrogen sulphide was concerned, the results were very markedly against the use of primary sodium

carbonate, as only one flask containing it showed even a trace of hydrogen sulphide produced. Moreover, when primary sodium carbonate was present, there was no greening of the solution or formation of dark green or greyish green sediment or cakes. The control flasks containing no primary sodium carbonate all produced hydrogen sulphide quickly and in large quantities, those containing potassium chloride giving the best results.

It is very pertinent to note here, even though it is anticipatory of the discussion concerning the reaction of the media, that the reaction of those media not containing primary sodium carbonate when they showed a trace of hydrogen sulphide production was about 0.70% normal acid on the average, using phenol phthalein as indicator, while the average reactions in the flasks which contained primary sodium carbonate was 1.68% normal acid when showing a like amount of hydrogen sulphide. It may be concluded from these figures that the supposition, that the presence of a carbonate would be beneficial by reason of the neutralizing effect which it would have upon the acid-reacting substances set free by the bacteria, thus removing from the field the byproducts of bacterial life which it was thought might have an inhibiting influence on the development of the bacteria and the consequent production of hydrogen sulphide, was an entirely erroneous one. Quite to the contrary, it proved that the bacteria must first neutralize this carbonate and then develope an acidity to phenol phthalein of 1.68% normal acid in the solution before they could begin breaking down the sulphur compounds of the peptone to yield hydrogen sulphide.

That no hydrogen sulphide had been produced and then held in solution as sodium sulphide, potassium sulphide, ferrous sulphide or calcium sulphide was demonstrated by acidifying a number of the cultures with hydrochloric acid, under which treatment, no appreciable amounts of hydrogen sulphide were evolved.

EFFECT OF THE INORGANIC SALTS CONTRIBUTED BY THE WATER UNDER EXAMINATION ON HYDROGEN SULPHIDE PRODUCTION.

It was thought that the inorganic salts which go to make up the total hardness in water, principally carbonates with some

sulphates, might have some influence on the amounts of hydrogen sulphide produced.

This question may be answered with the data already in hand without further experimental work.

Let us take an extreme case of a highly mineralized water. A sample taken from a spring near the laboratory showed 411.0 parts per million of total hardness in terms of calcium carbonate, with an alkalinity of 193.0 parts per million, also in terms of calcium carbonate.

Supposing that the alkalinity was all present as primary calcium carbonate, it would be equal to .0353% of primary calcium carbonate and equivalent to .0218% of calcium carbonate. Also supposing that the mineral acid hardness was all present as calcium sulphate, it would be equal to .0263% of calcium sulphate and equivalent to .0193% of calcium carbonate.

From the data and conclusions already given, it is evident that these percentages of calcium carbonate or primary calcium carbonate and of calcium sulphate are too small to have any effect upon hydrogen sulphide production, and consequently, the influence that the inorganic salts of water samples might have, may be entirely disregarded.

SUMMARY.

1. If sodium chloride is to be used in media for hydrogen sulphide production, not more than 1.5 percent of this salt should be present, as a larger amount has a decidedly inhibiting influence.

2. Cultures to which sodium chloride had been added in concentrations up to 1.5 percent, showed greater production of hydrogen sulphide than was observed in control cultures from which it had been omitted.

3. Sodium sulphate has an inhibiting effect upon hydrogen sulphide production in concentrations greater than 1.5 percent. In concentrations lower than that, it was found to be of slight assistance in augmenting hydrogen sulphide production, being inferior to sodium chloride in that respect.

4. The beneficial influence of potassium chloride on hydrogen sulphide production extends over a larger range in concentration

than was the case with any other salt tried, no inhibiting action occuring up to a 3.0 percent concentration.

5. In a 3.0 percent unadjusted peptone solution, the presence of from .5 percent to 1.0 percent of potassium chloride had a decidedly beneficial influence and helped to induce quicker and better results *than any other inorganic salt tried.*

6. As the concentrations of peptone and of acidity were decreased, the optimum concentration of potassium chloride increased. This would indicate that there is an increasing need of an ionizing salt to make the decreasing amounts of peptone available as food.

7. Magnesium chloride, calcium chloride and ammonium chloride in concentrations up to 2.0 percent had about equal influence upon hydrogen sulphide production, this influence being slightly beneficial.

8. Primary phosphates in concentrations of from 0.0 percent to 1.5 percent not only gave a large amount of turbidity in the culture, which is very undesirable, but had a decidedly inhibiting influence on hydrogen sulphide production.

9. Secondary phosphates in such concentrations as to furnish .05 percent, .15 percent and .25 percent of the basic elements present, showed an undesirable heavy white sediment, and as compared with the controls, the presence of secondary phosphates had an inhibiting influence on hydrogen sulphide production.

10. Tertiary phosphates had an even greater deleterious influence on hydrogen sulphide production than had primary and secondary phosphates.

11. During the course of the investigation, in which various chlorides, nitrates, sulphates and phosphates were used, many control cultures were prepared containing potassium chloride, sodium chloride and potassium sulphate in a concentration of .5 percent of the inorganic salt, and of these same salts in such concentrations as to furnish .05 percent, .15 percent and .25 percent of the basic elements, and also cultures containing no inorganic salt at all. A careful study and comparison of these check cultures shows that those containing an inorganic salt gave better and quicker results than those without inorganic salts; that the potassium salts were preferable to the sodium salts; that potas-

sium chloride produced the best results of any of the salts employed, in that it induced greater and better production of hydrogen sulphide, with greater uniformity of results in duplicate runs and cultures.

12. The presence of calcium carbonate in the cultures was found to be without effect upon hydrogen sulphide production.

13. The presence of primary sodium carbonate had an inhibiting influence on hydrogen sulphide production.

14. The amounts of inorganic salts which would be present in a water under examination are so small as to be negligable.

CHAPTER VI.

EFFECT ON HYDROGEN SULPHIDE EVOLUTION PRODUCED BY USING DIFFERENT BASES AND ACIDS IN ADJUSTING THE INITIAL REACTION TO PHENOL PHTHALEIN.

Although, after a large amount of data had been collected, it was found that just as good and in most cases, better, results were obtained when using an unadjusted 3.0 percent peptone medium, of which the reaction was always about 1.02 percent of normal acid ; still in the early part of the work, many experiments were tried to find the best reaction, and such bases as sodium hydroxide, potassium hydroxide, ammonium hydroxide, calcium oxide and magnesium oxide, and such acids as hydrochloric, sulphuric and nitric were used in adjusting the reaction to neutrality and also to various degrees of alkalinity and of acidity ; in combination with various salts and also with no added inorganic salts.

Without going into detail as regards the data collected in this connection in about five hundred inoculations, suffice it to say that sodium hydroxide and potassium hydroxide proved to be the best alkalies to use, with little choice between them, due presumably to the very small amounts of basic elements which were introduced in adjusting the reactions. Hydrochloric acid proved to be the best of the acids. Nitric acid, as would naturally be expected in a reducing action, proved a positive detriment.

SUMMARY.

Although the effect on hydrogen sulphide evolution produced by the use of different bases and acids in adjusting the initial reaction to phenol phthalein is very slight, potassium hydroxide and sodium hydroxide have been found to be the best bases and hydrochloric acid has been found to be the best acid to use.

CHAPTER VII.

EFFECT ON HYDROGEN SULPHIDE EVOLUTION OF THE INITIAL REACTION OF THE MEDIUM TO PHENOL PHTHALEIN.

While the use of other indicators than phenol phthalein may lead to results the interpretation of which may give valuable information, still, phenol pathalein has been used so universally in adjusting culture media that it seemed wisest to defer the study of other indicators to a later time and to use the one which has been almost entirely used by other investigators.

The questions of whether the temperature at which the titrations were made would make any difference in the results, and if so, at what temperature the titrations had best be made, and of whether the presence of inorganic salts would make any difference, naturally came up.

EFFECT OF TEMPERATURE ON REACTION.

It was found that the temperature at which the titration is made makes a very appreciable difference, as will be seen from the curves on Plates XVI and XVII, at the end of the article. Four points were determined on each curve, namely, at 3° Centigrade (as near 0° Centigrade as titrations could conveniently be made), at 20° Centigrade (practically room temperature), at 37½° Centigrade (the temperature of incubation), and at 95° Centigrade (as near the boiling point as titrations could conveniely be made).

Curve No. 1 shows the results for the initial reaction of media made up with 10 cubic centimeters of 30.0 percent peptone, 5 cubic centimeters of boiled, cooled and filtered tap water and 85 cubic centimeters of artificial sewage.

Curve No. 2 shows the results for the reaction after 24 hours incubation of the same inoculated media as in Curve No. 1.

Of course it must be understood that while the general form of Curve No. 2 will always hold for titrations made at different temperatures on media of the class which it represents, in which the particular flora of bacteria which was used in this experiment has been developing for 24 honrs, still its exact location on the chart will depend upon the amount of acid-reacting material which the bacteria have set free by hydrolytic cleavage.

It is of special interest to note that all of the solutions which were titrated above room temperature became redder and redder as they cooled. This serves to indicate that the apparently increased acidity at the higher temperatures is really due to an increased amount of ionization of the peptone, or of a temporary hydrolytic cleavage of the peptone at the higher temperatures, the cleavage products reuniting again as the temperature falls.

There is a general truth to be learned from the titrations made in this experiment ; namely, that in making culture media of any kind and for any purpose, attention should be paid to the temperature at which the media are titrated in adjusting the reactions of the same, otherwise, the chances are that one will not make two consecutive batches of media with the same reaction.

Therefore, having determined that the temperature at which the titration of media is made has a distinct influence, the question arises as to what temperature is best to use. This would appear to be largely a matter of convenience and of personal choice. Some might prefer 37½° as representing the temperature at which the bacteria would act upon the media in the incubator, but as 20°, room temperature, was more convenient to maintain while a large number of titration were being made, this was the temperature chosen in the present piece of work.

The essential thing is always to have the solutions to be titrated at the same temperature. While it is not as satisfactory a method of procedure, corrections for the temperature may be applied. For this purpose a curve has been plotted which was found to be practically smooth and hence a constant may be used for computing the reaction at one temperature from that found at another temperature. Under the conditions which were followed in this investigation ; that is, with a 3.0 percent, unadjusted, peptone solution, in round numbers the following formulas hold :—

The reaction at 3° C.	=	reaction at	20°	− .30%	normal	acid.
	=	" "	37½°	− .45%	"	"
	=	" "	95°	− .75%	"	"
The reaction at 20°	=	" "	3°	+ .30%	"	"
	=	" "	37½°	− .15%	"	"
	=	" "	95°	− .45%	"	"
The reaction at 37½°	=	" "	3°	+ .45%	"	"
	=	" "	20°	+ .15%	"	"
	=	" "	95°	− .30%	"	"
The reaction at 95°	=	" "	3°	+ .75%	"	"
	=	" "	20°	+ .45%	"	"
	=	" "	37½°	+ .30%	"	"

EFFECT OF SALTS ON REACTION.

No curves are shown for the initial reaction and the reaction after 24 hours of incubation of media containing potassium chloride, sodium chloride or potassium sulphate for the reason that these showed exactly the same variations with different temperatures as were shown in Curves No. 1 and No. 2; the inorganic salts mentioned having no effect in this respect and the curves for such media being identical with Curves No. 1 and No. 2.

When primary potassium phosphate was present, it was found that while it materially increased the acidity of the media; almost the same variations were found with different temperatures as in Curve No. 1 except that the curve flattened at the higher temperatures more than was the case when no phosphates were present.

Curve No. 3 shows the results for the initial reactions of media made up with 10 cubic centimeters of 30.0 percent peptone, 5 cubic centimeters of boiled, cooled and filtered tap water, .5 grams of primary potassium phosphate and 85 cubic centimeters of artificial sewage.

Curve No. 4 shows the results for the reactions of the same combination as in Curve No. 3, after 24 hours incubation.

Curve No. 5 is the curve for .5 grams of primary potassium phosphate in 100 cubic centimeters of boiled, cooled and filtered tap water and explains why the curve flattens out at the higher temperatures when primary phosphate is present in media.

OPTIMUM REACTION OF MEDIA.

It seems unnecessary to discuss in detail the experimental data with regard to any of the inorganic salts tried except potassium chloride, and therefore, the limits for the best concentrations of initial acidity for the other salts tried are given below in tabular form:—

For	$NaCl$,	from	0.0%	to	2.0%	N. acid;	best at	1.0%	at	20°	C.
"	Na_2SO_4,	"	.5	"	1.0	" " ;	" "	.5	"	"	
"	$MgCl_2$,	"	.5	"	1.5	" " ;	" "	1.0	"	"	
"	$CaCl_2$,	"	1.0	"	2.0	" " ;	" "	1.5	"	"	
"	NH_4Cl,	"	1.0	"	1.5	" " ;	" "	1.0	"	"	
"	K_2SO_4,	"	.5	"	2.0	" " ;	" "	1.0	"	"	

When peptone, potassium chloride, hydrochloric acid and potassium hydroxide were used in preparing the media, good results were obtained in some two hundred inoculations over a much wider field than with any other combination, as has already been noted; very good and quick results having been obtained with concentrations of acidity from .5 percent to 3.0 percent inclusive, but the very best results were obtained when the acidity was from 1.0 percent to 1.5 percent with very little choice between these concentrations, the preference, if any, being for 1.5 percent. This preference, however, was so slight that it is outweighed by the greater simplicity of leaving the reaction unadjusted at about 1.02 percent, when 10 cubic centimeters of 30.0 percent peptone is used in 100 cubic centimeters of inoculated media.

If the best concentration of the peptone were such as to give less than 1.0 percent of acidity when unadjusted, and if an adjustment were therefore made necessary, it would be advisable to make the reaction 1.5 percent of normal acid when potassium chloride is used in the medium; but, under the existing circumstances, 1.02 percent of acidity without adjustment is preferable on account of its greater simplicity.

SUMMARY.

1. The temperature at which peptone solutions are titrated has a very decided influence on the reaction when phenol phthalein is used as indicator; the higher the temperature the greater the amount of acidity found.

2. The reaction of a 3.0 percent unadjusted peptone solution, with or without potassium chloride, sodium chloride or potassium sulphate present, was found to be about one half at 3.0°, two thirds at 20.0°, and four fifths at 37.5° of what it was at 95.0°. This is believed to be due to the greater ionization or hydrolysis of peptone solutions at increased temperatures.

3. The reaction of peptone solutions is unaffected by the presence of potassium chloride, sodium chloride or potassium sulphate, but is affected by primary phosphates very materially.

4. The optimum initial reaction of the simple peptone medium, with or without potassium chloride, for hydrogen sulphide production is from 1.0 percent to 1.5 percent of normal acid, with a slight preference for the higher value. It makes but little difference whether this amount of acidity to phenol phthalein is contributed by an unadjusted peptone solution or whether the peptone is first neutralized with either potassium hydroxide or sodium hydroxide and then made acid with hydrochloric acid.

CHAPTER VIII.

RELATION TO HYDROGEN SULPHIDE EVOLUTION OF THE FINAL REACTION OF THE CULTURES TO PHENOL PHTHALEIN.

In the latter half of the work, titrations of the cultures after incubation, using phenol phthalein, were made, to ascertain whether there was any relation between the amounts of hydrogen sulphide produced and the reactions of the cultures.

Titrations were made of the unfiltered and of the filtered media; and also, at first, titrations were made at 3.0°, 20°, 37½° and 95° Centigrade, just as in the case of obtaining the initial reactions.

EFFECT OF TEMPERATURE ON REACTION.

The question of titrations at different temperatures may be dismissed by refering to Curve No. 2 presented on Plate XVI at the end of the article, which is typical of all of the results which were obtained at different temperatures and which showed almost exactly the same variations due to difference in temperature as were shown in the case of the initial reactions. This is in entire accord with what might be expected, for the variations in reaction with variations in temperature are due to the peptone, of which only a small amount out of the total present is broken down during the incubation of the cultures, leaving almost as much undecomposed peptone to cause the variations after incubation as before.

As the same variations in reaction with different temperatures were found after incubation as before, and as 20° Centigrade was chosen as the temperature for making the titrations of initial reaction, the same temperature was naturally chosen for making the titrations of final reaction.

DEDUCTIONS DRAWN FROM FINAL REACTION.

The observations which follow are more or less fragmentary and incomplete for the reason that these observations were made on inoculations which were parts of runs primarily planned to obtain information on entirely different questions from that now

being discussed, but still it is deemed wise to include the data even though the observations are not all logically related, for the reasons :—first, that the amount of acidity finally developed in the cultures is a function of the activity and amount of growth and developement of the bacterial flora present ; second, that it gives a different basis for studying the effect of adding different inorganic salts to the media ; and third, that some really valuable deductions may be drawn from the observations which were made, these deductions being as follows :—

1. With varying amounts of potassium chloride, sodium chloride and potassium sulphate added separately to the media, those media which received potassium chloride gave the most uniform amounts of increase in acidity, just as potassium chloride induced the most uniform results as regards hydrogen sulphide production.

2. The data for increases in acidity after 24 hours of incubation, with potassium chloride, sodium chloride or potassium sulphate present in concentrations furnishing equal amounts of basic element, namely, .25 grams, are rather meagre, only three observations having been made with each salt, but the observations were made at different periods of time and on inoculations made with different samples of sewage and hence are worthy of note. When potassium chloride was present, an average increase over the original acidity of .72 percent of normal acid was noted ; for potassium sulphate, an average increase of .65 percent was noted and for sodium chloride, an average increase of .42 percent was noted, resulting in respective average reactions of 1.77 percent, 1.70 percent and 1.47 percent. This is in accord with the fact that the quickest results as regards hydrogen sulphide production were obtained in the presence of potassium chloride.

3. In 48 hours of incubation, even with amounts of sewage varying from 5 cubic centimeters to 85 cubic centimeters, little variation in the amounts of acidity produced were noted when potassium chloride, sodium chloride or potassium sulphate were present, the final reaction being in all cases close to 3.0 percent of normal acid ; the average for nineteen inoculations in which .25 grams of potassium as potassium chloride was used, being 3.17 percent ; for nineteen inoculations in which .25 grams of sodium as sodium chloride was used, being 3.03 percent ; and for

nineteen inoculations in which .25 grams of potassium as potassium sulphate was used, being 2.72 percent; showing potassium chloride to be slightly superior to sodium chloride or potassium sulphate in furnishing the best conditions for the general growth and development of the bacteria, just as it furnished the best conditions for the activity of that part of the flora which produced hydrogen sulphide. The largest amount of acidity noted was produced in the presence of potassium chloride but this fact is not considered to be of much importance as it was undoubtedly due to the presence of very active organisms in that particular inoculation. As has been before stated, in work of this kind where a mixed flora is being used one must depend upon the averages of large numbers of runs and not upon single observations or groups of observations which are either small in number or all made at one time from one particular sample.

4. With .25 percent of potassium as potassium chloride present, more acidity was developed in both 24 and 48 hours than was produced when peptone alone without added salt was used. For peptone alone, average increases of .57 percent and 1.91 percent were found for 24 and 48 hours respectively as against .72 percent and 2.12 percent when potassium chloride was present, showing that potassium chloride has a beneficial influence upon the general development of the whole bacterial flora which was present in this investigation as well as upon hydrogen sulphide production.

5. In the presence of primary phosphate, the development of acidity was very slight indeed.

6. Tertiary phosphates had an inhibiting effect upon the development of acidity.

7. The presence of secondary phosphates in some instances led to a greater development of acidity than was the case when peptone alone was used. This was a circumstance which was noted with pleasure as it would seem to constitute a justification of their use in so much of the present day media, although it is still maintained that they be omitted from media for hydrogen sulphide production so long as peptone is used in the media. It may be that when we find a synthetic compound to take the place of peptone in the medium, as we hope to do, that we will find with

Frouin[95] that some form of phosphate is absolutely indispensable in media which contain no protein ; but when peptone is present, the phosphates do undoubtedly interfere with hydrogen sulphide production.

8. The presence of calcium carbonate had a beneficial effect in inducing the production of a greater increase in acidity in both 24 and 48 hours in those media which were titrated, and hence, we see its justification in some kinds of media even though it did not seem to have any beneficial influence on hydrogen sulphide production.

9. Those cultures to which no sewage was added did not change in reaction after sterilization or after incubation at 37½° even for 72 hours, and hence it may be stated that the application of heat does not have the effect of breaking down the peptone permanently to give a greater amount of acidity to phenol phthalein.

10. Much of the acid-reacting substances is in the sediment produced in the cultures, as the filtrate from such cultures showed a markedly lower acidity than cultures which were unfiltered but which were of the same initial composition and which had been treated in the same way in all other respects. As the whole culture and not the filtrate is of special interest in this investigation, all of the foregoing deductions are based on unfiltered cultures.

NOTE ON THE SPEED OF EVOLUTION OF HYDROGEN SULPHIDE.

During the course of the work, and more especially, while making titrations of some of the media after incubation, it was noted that there would be at times a very rapid evolution of hydrogen sulphide. This never occurred during the first twenty-four hours of incubation, and never, as far as observed, before the cultures had reached an acidity of 3.0 percent.

The evolution was so rapid at times that from one-quarter to one-third of the lead acetate papers in the tubes of the culture flasks would be blackened in the half hour or so that it took to titrate a group of fifteen or twenty cultures.

It was also noted that the same sort of thing occurred in the incubator in short periods of time.

The indication is that exactly the right conditions must be inaugurated by the bacteria and that then hydrogen sulphide production proceeds rapidly.

SUMMARY.

1. The same variations with temperature were noted after inoculation and incubation of cultures as before.

2. A study of the reactions of the media after inoculation and incubation furnishes excellent corroboration of the conclusions already drawn concerning hydrogen sulphide production, on the assumption that the amount of acidity produced is a measure of the activity and of the amount of growth and development of the bacterial flora present. From such a study the following deductions may be made :—

(a) With varying amounts of potassium chloride, sodium chloride and potassium sulphate added separately to the media, those media that had received potassium chloride gave the most uniform amounts of increase in acidity.

(b) At the time when hydrogen sulphide was first being produced, an average acidity of about 2.0 percent was found, this acidity increasing with increased production of hydrogen sulphide, until an average acidity near 3.0 percent was reached, when most abundant production of hydrogen sulphide was noted.

(c) Cultures containing potassium chloride gave, on the average, larger increases in acidity in both twenty four and forty eight hour incubations than did cultures containing either sodium chloride or potassium sulphate, and cultures with any of these salts present gave larger increases in acidity than cultures containing no added salt.

(d) In the presence of primary phosphates, the development of acidity was very slight.

(e) Tertiary phosphates had an inhibiting effect upon the production of acidity.

(f) The presence of secondary phosphates in some instances led to a greater development of acidity than was noted with peptone alone. This would seem to justify their use in certain classes of culture media.

3. More acid-reacting material is in the sediment of the cultures than in the soluble part.

CHAPTER IX.

THE METHOD FOR DETECTING THE BACTERIA PRODUCING HYDROGEN SULPHIDE AS IT HAS BEEN FOUND TO GIVE THE MOST RAPID AND RELIABLE RESULTS.

To make one liter of the culture medium, bring 700 cc. of tap water to a boil and add 300 grams of Witte peptone and 75 grams of potassium chloride. Maintain a gentle heat and stir constantly until as much of the peptone as will do so, has gone into solution. Cool rapidly by immersing the vessel containing the medium in cold water. Make up to one liter with tap water. Transfer to a flask, bring to a boil, plug the flask immediately and cool again. Place in the ice box for at least twenty four hours. Filter the cold medium through paper and measure 10 cc. portions of the clear filtrate into the desired number of the special culture flasks illustrated in Cut 1 at the end of the article, which have been provided with tiny cotton plugs in the bottom of the tube of the caps. Sterilize the flasks in the autoclave at one atmosphere, and after they are cool, place in each tube a strip of bibulous paper measuring 25 mm. by 2 mm. which has previously been impregnated with neutral lead acetate and dried. Finally, apply a tight tin foil cap to the top of the tube. This tin foil cap is made by winding a piece of tin foil measuring about 40 mm. by 20 mm. around the top of the tube in the form of a cylinder, leaving about one half of the cylinder (10 mm.) projecting above the tube. The part which projects is then twisted into a tight twist and the flasks are ready for either storage or inoculation. (That part of the medium not introduced into the special culture flasks may be sterilized and kept for future use.)

To inoculate, remove cap from flask and pour in the sample under examination until the 100 cc. mark is reached, replace the cap, mix thoroughly by rotating the flask and incubate at from 37 to 38 degrees Centigrade.

Examine the cultures at the end of 18 hours and as frequently thereafter as possible, as the length of time required for development of hydrogen sulphide may be taken as a rough measure of

the degree of activity, or of the number, of the putrefactive organisms present, and hence as an indication of the age and extent of the pollution.

Besides noting the amount of hydrogen sulphide produced, as measured by the amount of blackening of the lead acetate paper, the degree of turbidity of the solution should be noted, the color of the solution (whether it has been turned greenish or not), the presence and color of any sediment formed, the presence and color of any floating cakes of material, and the presence of gas bubbles on the liquid. The amount of indol present may also be determined as corroborative evidence.

SUMMARY.

The method which has been found to give the most rapid and reliable results is very easy to follow and the medium is simple to make.

CHAPTER X.

THE SENSITIVENESS AND RELIABILITY OF THE METHOD.

WITH ARTIFICIAL SEWAGE.

In order to try out the method with waters of a varying degree of pollution, an artificial sewage was prepared and plated on standard gelatine and on standard agar and also was inoculated into fermentation tubes containing a 2.0 percent dextrose, 3.0 percent peptone, .5 percent potassium chloride medium.

After 48 hours of incubation, the gelatine plates showed 2,800 colonies per cc., with 20 *B. coli communis* per cc. and the agar plates showed 200 colonies per cc. Of the fermentation tubes, those which had been inoculated with 1 cc. and with .1 cc. had one half of the closed arms filled with gas, but those inoculated with .01 cc. and .001 cc. showed no signs of gas or growth.

For inoculating the media for the detection of hydrogen sulphide, dilutions of the sewage of 5:100, 10:100, 15:100, 20:100, 25:100, 30:100, 35:100, 40:100, 45:100, 50:100, 55:100, 60:100, 65:100, 70:100, 75:100, 80:100 and 85:100 were made with sterile tap water, and the flasks inoculated with these dilutions were incubated at from 37 to 38 degrees Centigrade.

There was, as would be expected, a gradual increase in the amounts of hydrogen sulphide produced and also in the rapidity with which it was produced as the concentration of the sewage was increased, but after 18 hours of incubation, the sewage which had been reduced to 85:100 had given an appreciable amount of hydrogen sulphide with a turning green of the medium and turbidity produced therein; and after 30 hours, much hydrogen sulphide had been produced with a dark green sediment and dark green floating cakes and an accumulation of gas at the top of the solution by even the 10:100 dilution; so it may be concluded that a positive test will be given in 18 hours with a 90 cc. inoculation of a water which would show 2,380 colonies per cc. on standard gelatine with 17 *B. coli* per cc.; 170 colonies on standard agar; and gas in dextrose, peptone, potassium chloride medium in fermentation tubes with a 1. cc. inoculation; while a positive test

will be given in 30 hours with a water containing only about one eighth this amount of pollution, with a proportionate amount of time required by degrees of pollution between these extremes.

These figures are well within the limits of what will be found in sewage polluted waters and hence this method of detecting sewage pollution in a water by means of the hydrogen sulphide produced in the proposed medium by the putrefactive organisms, affords a very valuable method for augmenting and helping to interpret the data obtained by the chemical and bacteriological examinations of water samples.

WITH NATURAL WATER SAMPLES.

Fairly complete chemical and bacteriological examinations of a large number of water samples have been made with the following results :—

1. *A badly polluted water.*

The sample was taken from a dug well near the University, of loose stone construction so that surface water could find entrance.

CHEMICAL EXAMINATION.

Total solids	450.	parts per million.
Loss on ignition	107.	
Mineral residue	343.	
Nitrogen as total ammonia	.038	
Nitrogen as free ammonia	.015	
Nitrogen as albuminoid ammonia	.023	
Nitrogen as nitrites	.020	
Nitrogen as nitrates	1.900	
Oxygen consumed	.600	
Chlorine	19.200	
Alkalinity (as $CaCO_3$)	247.	
Mineral acid hardness (as $CaCO_3$)	105.	
Total hardness (as $CaCO_3$)	352.	

BACTERIOLOGICAL EXAMINATION.

Standard gelatine, incubated at 20° for 48 hours, 3600 colonies.

Standard agar, incubated at 37° for 48 hours, 260 colonies.

Dextrose peptone, incubated at 37° for 24 hours, gas and turbidity from 1. cc. inoculation.

Phenolized dextrose peptone, incubated at 37° for 24 hours, gas and turbidity from 1 cc. inoculation.

Dextrose peptone, lactose litmus peptone, and lactose neutral red fermentation tubes all showed from 50% to 85% of gas with a gas ratio of 1 : 1.7 in all tubes inoculated with .1 cc.

Test for Putrefactive Organisms, incubated at 37° for 24 hours, showed the lead acetate paper entirely blackened, the medium turbid and green in color, with dark green sediment and dark green floating cakes. Much indol.

2. *A good water.* The sample was taken from a cased, driven well.

CHEMICAL EXAMINATION.

Total solids	310.	parts per million.
Loss on ignition	84.	
Mineral residue	226.	
Nitrogen as total ammonia	.028	
Nitrogen as free ammonia	.016	
Nitrogen as albuminoid ammonia	.012	
Nitrogen as nitrites	.0035	
Nitrogen as nitrates	.125	
Oxygen consumed	.925	
Chlorine	3.000	
Alkalinity (as $CaCO_3$)	263.5	
Mineral acid hardness (as $CaCO_3$)	16.	
Total hardness (as $CaCO_3$)	279.5	

BACTERIOLOGICAL EXAMINATION.

Standard gelatine, incubated at 20° for 48 hours, 375 colonies.
Standard agar, incubated at 37° for 48 hours, 2 colonies.
Dextrose peptone, incubated at 37° for 24 hours, turbid, no gas.
Phenolized dextrose peptone, incubated at 37° for 24 hours, sterile.
Lactose, neutral red fermentation tubes, incubated at 37° for 48 hours, sterile.
Test for Putrefactive Organisms, incubated at 37° for 24 hours, slight turbidity but no evidence of hydrogen sulphide. No indol. No change after 72 hours.

3. *A suspicious water.*

The sample was taken from a driven well about five miles north of Ithaca on Cayuga Lake, the well being 100 feet from a privy.

BACTERIOLOGICAL EXAMINATION.

Standard gelatine, incubated at 20° for 48 hours, 50 colonies.
Standard agar, incubated at 37° for 48 hours, 8 colonies.
Harrison agar, incubated at 37° for 48 hours, negative.
Phenolized dextrose peptone, incubated at 37° for 24 hours, no gas or turbidity from 5. cc. or 10. cc. inoculations.
Lactose neutral red peptone fermentation tubes, incubated at 37° for 48 hours, slight growth, acid ; no gas from 5. cc. or 10. cc. inoculations.
Test for Putrefactive Organisms, incubated at 37° for 24 hours, no trace of hydrogen sulphide ; after 72 hours of incubation there was a slight amount of light gray sediment, no floating cakes, no gas, a slight amount of hydrogen sulphide and a slight test for indol.

4. In Table IV, is given a summary of 141 samples of water which have been received at the laboratory from various sources for analysis and on which the proposed method for the detection of putrefactive organisms has been employed.

The character "X" in the table represents a slight amount, "XX" represents a medium amount, and "XXX" represents a large amount. "Tr." represents a trace only.

A careful study of the data in this table will show that the test for hydrogen sulphide was practically never positive in 24 hours in a really satisfactory water and vice versa.

In many instances, it was the hydrogen sulphide test which gave the clue to why the chemical character was poor when there was no gas production and no *B. coli* were found.

TABLE IV.

Sample No.	Chemical character.	Bacteria per cc.	Gas production.	B. Coli.	Hydrogen sulphide.	Indol.
		SURFACE WATERS.				
428	Poor	460	X	X	XX	X
449	Bad	600	X	X	XXX	XX
450	Bad	1600	X	X	XX	XX
452	Suspicious	400	X	X	X	X
464	Poor	1600	X	X	X	XX
489	Good	50	X	o	XX	X
544	Suspicious	3100	X	?	XX	XX
545	Suspicious	8	o	o	X	X
548	Bad	50000	X	X	XX	XX
549	Poor	5000	X	?	X	X
555	Bad	46000	X	X	X	X
556	Good	460	o	o	o	o
561	Poor	2200	o	o	XX	X
566	Poor	50000	X	X	XX	X
568	Poor	300	o	o	X	o
		WELLS.				
429	Very high Cl and solids	720	X	?	X	XX
440	Good	35	o	o	o	Tr.
443	Good	330	X	?	X	X
447	Suspicious	130	X	?	X	o
448	Poor	10	X	X	XX	X
455	Good	6	o	o	o	o
472	Bad	225	X	X	XX	X
483	Good	50	o	o	o	o

TABLE IV.—Continued.

Sample No.	Chemical character.	Bacteria per cc.	Gas production	B. Coli.	Hydrogen sulphide.	Indol.
484	Good	5	O	O	Tr.	O
485	Good	7	O	O	O	O
486	Good	3	O	O	O	O
487	Good	6	O	O	O	O
488	Good	2	O	O	O	O
516	Poor	1500	X	X	XX	X
528	Poor	70	O	O	X	O
535	Bad	300	X	O	X	O
536	Good	39000	X	?	XX	XX
550	Suspicious	1500	X	?	XX	XX
552	Good	50	O	O	O	X
569	Bad	3000	X	O	XX	O
572	Poor	2000	X	X	X	O
575	Poor	2000	X	X	XX	X
583	Good	250	X	?	XX	X
584	Suspicious	145000	X	X	XXX	X
585	Good	6500	O	O	O	O
589	Good	750	X	X	O	O
590	Good	480	X	O	O	O
2012	Poor	100	X	X	XX	XX
2013	Good	270	O	O	O	O
2069	Good	2000	X	O	O	O
		SPRINGS.				
430	Good	50	O	O	O	Tr.
490	Good	10	O	O	O	O
515	Good	30	X	X	XX	X
524	Good	65	X	?	X	X
526	Bad	145	X	?	O	O
527	Bad	10	O	O	O	O
537	Good	40	X	X	O	O
539	Bad	210	X	?	XX	X
546	Good	180	O	O	O	O
547	Good	16	O	O	O	O
551	Good	40	O	O	O	O
557	Good	7200	O	O	XX	X
558	Good	110	O	O	O	O
559	Good	200	O	O	O	O
570	Good	135	O	O	O	O
573	Good	35	O	O	O	O
574	Poor	350	X	X	X	X
582	Suspicious	500	O	O	O	O
586	Good	600	X	X	O	O
2015	Good	800	O	O	X	X
2064	Good	9	X	O	O	O

TABLE IV.—Continued.

Sample No.	Chemical character.	Bacteria per cc.	Gas production.	B. Coli.	Hydrogen sulphide.	Indol.
		MISCELLANEOUS.				
2080	Suspicious	3500	X	?	XXX	X
2080a	Good	350	X	?	XX	X
2083	Bad	3500	o	o	XX	X
2085	Good	95	X	?	XX	X
2086	Bad	5000	X	X	XX	X
2087	Bad	500	o	o	o	o
2088	Suspicious	1000	X	?	XX	X
2089	Good	37500	o	o	o	o
2091	Bad	9000	X	X	XXX	XX
2091a	Bad	5000	X	X	XXX	XX
2094	Bad	600	X	o	X	X
2097	Suspicious	50000	X	X	XXX	XX
2098	Good	18000	X	X	XX	o
2109	Good	500	X	o	X	o
2110	Suspicious	1500	X	X	XX	XX
2111	Bad	2100	X	X	XXX	XX
2112	Bad	37500	X	X	XX	X
2113	Good	15000	o	o	o	o
2116	Suspicious	1800	X	o	X	o
2117	Good	3800	X	X	XX	X
2119	Good	390	X	o	X	X
2120	Good	1400	X	o	XX	X
2121	Good	1500	X	?	o	o
2122	Good	10000	X	X	XX	X
2124	Good	3	o	o	o	o
2126	Good	1000	X	o	XXX	XX
2127	Suspicious	500	X	o	XX	X
2129	Poor	15	o	o	X	X
2130	Good	100	X	X	o	o
2131	Suspicious	1500	o	o	X	X
2132	Suspicious	5000	X	o	X	X
2133	Suspicious	1500	o	o	XX	X
2134	Suspicious	1000	o	o	XX	o
2136	Suspicious	1200	X	o	XX	X
2137	Good	6000	o	o	X	X
2138	Bad	4500	o	o	XX	X
2139	Good	35	o	o	o	o
2140	Bad	18500	o	o	XX	X
2141	Good	75000	X	o	XXX	X
2142	Good	540	o	o	XX	XX
2143	Good	250	X	o	X	X
2144	Good	270	X	o	X	X

TABLE IV.—Continued.

Sample No.	Chemical character.	Bacteria per cc.	Gas production.	B. Coli.	Hydrogen sulphide.	Indol.
2145	Good	25000	o	o	o	o
2148	Good	370	X	o	XX	X
2149	Good	20	X	?	o	o
2150	Suspicious	5500	X	X	XX	XX
2151	Suspicious	500	X	X	XX	XX
2154	Good	725	X	X	X	XX
2155	Bad	120	o	o	XX	X
2156	Bad	550	X	X	XXX	X
2157	Bad	130	X	X	XX	XX
2158	Suspicious	70	X	?	XX	XX
2159	Bad	5000	X	?	XXX	XX
2160	Bad	2000	o	o	XX	X
2161	Good	15	X	o	o	o
2162	Good	6000	o	o	o	o
2165	Suspicious	4000	X	o	XX	XX
2166	Bad	7000	X	?	XXX	XX
2168	Suspicious	250	o	o	o	o
2169	Suspicious	3100	X	X	XX	XX
2170	Suspicious	180	o	o	o	o
2171	Suspicious	140	o	o	XX	X
2175	Bad	1000	X	X	XXX	XX
2176	Good	450	o	o	o	o
2178	Bad	170000	o	o	o	o

SUMMARY.

1. Positive results are obtained by this method in 18 hours with sewage polluted waters showing an amount of pollution often met in actual work.

2. No hydrogen sulphide is produced in as long a period of incubation as 72 hours with natural waters of good quality when inoculated into the peptone, potassium chloride medium; while much hydrogen sulphide is produced in as short a period as 18 hours with natural waters of bad quality, with a nicety of gradation in results corresponding to the quality of the water.

CHAPTER XI.

A COMPARATIVE STUDY OF METHODS FOR THE QUANTITATIVE DETERMINATION OF SULPHUR IN PEPTONE.

As a preliminary step to making quantitative determinations of the amount of sulphur in the culture media, both before and after inoculation with sewage or water samples, and before and after incubation, a comparative study was made of those methods for the determination of sulphur in organic compounds which a search of the literature showed as having given the best results in the hands of other investigators. Witte peptone was used as the sulphur-containing material and the determinations were made in triplicate in each case, using 2 gram portions of peptone.

METHODS FOR TOTAL SULPHUR.

Liebig Method.

The method of Liebig, as described in Organic Analysis, Sherman, page 298, 1912 edition, was first tried.

In a large nickel crucible, 8 grams of potassium hydroxide and 1 gram of potassium nitrate were mixed and fused over an alcohol lamp, stirring almost constantly. The fused mass was allowed to cool and exactly 2 grams of finely pulverized peptone was added. The mass was heated gently and as soon as it became soft it was well mixed. The heat was gradually increased, the mass being constantly stirred. The final heating was done over a Bartel alcohol burner. The perfectly clear fusion was allowed to cool, dissolved in water in a Jena glass beaker, acidified with hydrochloric acid, evaporated to dryness, heated to 110° for four hours in an air bath in order to dehydrate any silica present, dissolved in cold water, 5 cc. of hydrochloric acid added, the solution filtered, heated to boiling and the sulphate precipitated with barium chloride in the usual manner.

Another group of determinations was made by the Liebig method in which a much lower heat and greater amounts of potassium nitrate were employed than above.

Osborne Method.

The method of Osborne, as described in the Journal of the American Chemical Society, 24, 142 (1902) was tried.

Ten grams of sodium peroxide was converted into sodium hydroxide by adding to it, in a large nickel crucible, a slight excess of water. Heat from an alcohol lamp was applied until

the excess of water was boiled off. The fusion was allowed to cool until pasty when 2 grams of peptone was added and rapidly stirred in with a platinum spatula. After the first vigorous action was over the heat was increased until the mass fused completely, when 5 grams of sodium peroxide in small portions was added ; the final heating being done over a Bartel alcohol burner until the fusion was perfectly clear. The fused mass after cooling, was dissolved in water, acidified with hydrochloric acid and from this point treated exactly as in the Liebig process.

A. O. A. C. Method.

The A. O. A. C. method, as described in U. S. Dept. of Agriculture, Bureau of Chemistry, Bulletin 107, Revised, page 23, was next tried.

Two grams of peptone was placed in a large nickel crucible and was moistened with 2 cc. of water and thoroughly mixed with a platinum spatula. Five grams of sodium carbonate was added and mixed in. Five grams of sodium peroxide in small portions was thoroughly mixed in. The mass was slowly brought to a state of fusion over an alcohol lamp while being stirred. It was allowed to cool, 5 grams of sodium peroxide was added and the heat was gradually increased until the mass was completely fused. The final heating for ten minutes was done over a Bartel alcohol burner. The fused mass was allowed to cool, dissolved in water, acidified with hydrochloric acid and from this point treated exactly as in the Liebig process.

Liebig—Koch Method.

The Koch modification of the Liebig method as described in Chem. Centr., 1886, 894, was next tried.

Two grams of peptone was placed in a Jena glass beaker and 20 cc. of nitric acid having a specific gravity of 1.4 was added. An energetic reaction was noted with the liberation of much nitrogen tetroxide. The mixture of peptone and nitric acid was allowed to digest for two hours on the water bath, the beaker being covered with a watch glass during the digestion. Then the watch glass was removed and the nitric acid was evaporated on the water bath. Twenty cubic centimeters of distilled water was added and the material again evaporated to dryness after which the addition of water and evaporation was repeated. The material was then dissolved in hot water and transferred to a large nickel crucible and evaporated again to dryness. Eight grams of potassium hydroxide and 1 gram of potassium nitrate were then added and the operations of the regular Liebig method, already described, were carried out.

After one or two trials of the method as described above, it was modified as follows :—

The solution of the material, after the nitric acid treatment, was introduced into a nickel crucible containing eight grams of potassium hydroxide and about one half gram of potassium nitrate and then evaporated over an alcohol lamp while a jet of air was allowed to impinge on the surface of the liquid. This resulted in a homogeneous mixture of the material under examination with the hydroxide and nitrate, thus precluding the tendency to burn which the peptone displayed in earlier determinations. Another device, trivial but nevertheless, worthy of note, was used in connection with the transference of the material which had been treated with nitric acid to the nickel crucible. A number of particles difficulty soluble in hot water clung tenaciously to the sides of the beaker. Obviously a rubber-tipped rod could not be used to loosen them because of the danger of minute particles of the rubber becoming detached which might contribute appreciable amounts of sulphur. Therefore, a small piece of filter paper on the end of a glass rod was employed for scrubbing. This effectively loosened up all particles. The piece of filter paper with any adhering material was introduced into the nickel crucible and was of course destroyed during the alkali-nitrate fusion.

It was deemed advisable to ascertain whether any loss of sulphur in volatile form might take place during the treatment with nitric acid in the Liebig-Koch method.

For this purpose, a 250 cc. ether wash bottle was modified by cutting off the tube, which extended down into the bottle, a little way above the stopper and fusing on a small separatory funnel. The lower end of this tube was drawn out and bent into a shepherd's crook. To the other tube, which emerged from the stopper of the wash bottle, was fused a piece of glass tubing which was bent so as to nearly reach to the bottom of a Fritz Friedrichs gas wash bottle, the connection being made by means of a soft-rolled cork stopper. The glass tube which was fused into the stopper of the Fritz Friedrichs wash bottle was connected with a suction pump by means of a rubber tube upon which was placed a screw clamp to facilitate the regulation of the amount of suction employed.

Two grams of peptone was placed in the ether wash bottle which was mounted on a boiling water bath and connected to the Fritz Friedrichs wash bottle, into which 250 cc. of 12 per-

cent potassium hydroxide had been introduced. Suction was applied and 20 cc. of nitric acid having a specific gravity of 1.4, was introduced through the separatory funnel. The suction was so regulated that about two bubbles of air per second were drawn through the apparatus, the air entering through the separatory funnel. Thus, any volatile compounds of sulphur which might be liberated during the process would be drawn into the potassium hydroxide solution in the wash bottle and held there as the potassium salt.

It required two weeks, running eight to ten hours a day, to completely distil all of the nitric acid from the ether wash bottle into the Fritz Friedrichs wash bottle.

The material in the ether wash bottle was then dissolved in hot water and transferred to a Jena glass beaker, acidified with hydrochloric acid, evaporated to dryness on a water bath, 20 cc. of distilled water added, again evaporated to dryness, heated for 4 hours in an air bath at 110°C. to dehydrate the silica, dissolved in water, 5 cc. of hydrochloric acid added, filtered, and the sulphur existing as sulphuric acid, precipitated and weighed in the usual manner as barium sulphate.

For comparison, the amount of sulphur existing as sulphuric acid after treatment of peptone with nitric acid in open beakers was determined.

For this purpose, 2 gram portions of peptone were placed in Jena glass beakers, 20 cc. of nitric acid, having a specific gravity of 1.4, was added to each, and digestion was carried on for two hours on a water bath ; each solution was then evaporated to dryness, 20 cc. of distilled water added, again evaporated to dryness, heated for 4 hours in an air bath at 110°C., dissolved in water, 5 cc. of hydrochloric acid added, filtered, and the sulphur existing as sulphuric acid precipitated and weighed in the usual manner as barium sulphate.

The close agreement between the results obtained in the closed system and in the open beakers, while not proving that there is no loss of volatile sulphur compounds, does prove that the length of time of the digestion with nitric acid above two hours has no effect, as the nitric acid was in contact with the peptone for two hours in the one case and for two weeks in the other.

To ascertain whether any loss had taken place during the nitric acid treatment in the closed system, the amount of sulphur

in the solution contained in the Fritz Friedrichs wash bottle was determined.

For this purpose, the solution ; which was calculated to contain 30 grams of potassium nitrate resulting from the neutralization of the 18.3 grams of nitric acid distilled over from the ether wash bottle plus 14. grams of potassium hydroxide remaining from the 30. grams originally introduced in the form of 250 cc. of 12 percent potassium hydroxide, together with any potassium sulphide, potassium sulphite or potassium sulphate which might have been formed ; was transferred to a large nickle crucible, evaporated to dryness and fused, in order that any sulphide or sulphite might be oxidized to sulphate. The fused mass was dissolved in water, an excess of hydrochloric acid added, evaporated to dryness, 20 cc. of distilled water added, again evaporated to dryness, heated for 4 hours in an air bath to 110° C. to dehydrate the silica, dissolved in water, 5 cc. of hydrochloric acid added, filtered, and precipitated with barium chloride in the usual manner.

The results showed that there was no formation of volatile sulphur compounds during the nitric acid digestion in the closed system ; and since the results in the open beakers were in such close agreement with those obtained in the closed system in the matter of the percent of sulphur revealed by nitric acid digestion, it is safe to assume that no loss of sulphur as volatile compounds occurs during nitric acid digestion in open beakers.

Petersen Method.

Since Petersen (Zeit. anal. Chem., 42, 406) had made the statement that the sulphur of certain organic compounds may be quantitatively oxidized to sulphuric acid by means of hydrogen peroxide in alkaline solution, it was decided to try the effect of perhydrol (a 40% solution of hydrogen peroxide) with which it was expected to get very complete and rapid oxidation of the organic sulphur compounds. Without giving the details of the methods employed, suffice it to say that, dissolving the peptone in water and then boiling with perhydrol ; dissolving the peptone in alkaline solution and then boiling with perhydrol ; digesting the peptone with nitric acid having a specific gravity of 1.4, and then boiling with perhydrol ; were all tried with little success, the greatest amount of sulphur, after making all necessary corrections, which was found was .5938 percent, as the result of the method in which a preliminary digestion with nitric acid was made. Without this preliminary digestion, .3025 percent of

sulphur was found. In other words, only from one third to one half of the sulphur actually present was oxidized to sulphate. Further work on this method is needed.

Parr Method.

The determination of the total sulphur by means of the Parr calorimeter was next tried.

For each charge, a mixture of 1. gram of peptone, 15. grams of sodium peroxide and 1. gram of potassium chlorate was employed. A hot fragment of iron wire was used to start the ignition. After the charge had been ignited it was in each case dissolved in hot water, acidified with hydrochloric acid, evaporated to dryness, again acidified, evaporated, heated to 110° for 4 hours to dehydrate the silica, dissolved in hot water, acidified with hydrochloric acid, filtered and precipitated in the usual manner with barium chloride.

METHODS FOR PART OF SULPHUR ONLY.

Attention was next turned to methods for determining a part of the sulphur only.

Schultz Method.

First, the Schultz method described by Osborne in the Journal of the American Chemical Society (1902) 24, 140, for the determination of loosely bound sulphur, was tried.

Two grams of peptone was introduced into a round bottom flask of 300 cc. capacity. To this was added 100 cc. of a 30.% sodium hydroxide solution, 2. grams of metallic zinc and a little lead acetate. This mixture was then boiled for five hours with a reflux condenser, after which it was acidified with acetic acid and the lead sulphide formed, was filtered, washed with water, dried, and fused with sodium peroxide and sodium hydroxide. The fusion was dissolved in water, its solution treated with carbon dioxide to remove lead, filtered, evaporated with an excess of hydrochloric acid, dehydrated and the sulphur determined as sulphate in the usual way.

Nitric Acid—Potassium Chlorate Method.

The effect of a saturated solution of potassium chlorate in nitric acid was tried next upon peptone for the reason that it was believed that this reagent would give a measure of the *easily oxidized sulphur* present.

The method consisted in digesting 2. grams of peptone contained in a Jena glass beaker with 20 cc. of a saturated solution of potassium chlorate in nitric acid on a water bath for two hours, then evaporating to dryness, adding 5 cc. of hydrochloric acid and 20 cc. of water and evaporating to dryness, dehydrating, and determining the sulphur as sulphate in the usual way.

Hydrochloric Acid—Potassium Chlorate Method.

Determinations were made using potassium chlorate and hydrochloric acid as the oxidizing agent.

SULPHUR IN UNTREATED PEPTONE.

The amount of sulphur precipitable as barium sulphate in a solution of peptone which had not been treated in any way was determined.

From 2 gram portions of peptone, precipitates of barium sulphate weighing .0054 and .0052 grams were obtained.

EXTRACTION OF PEPTONE WITH ALCOHOL.

It seemed desirable to ascertain the percent of sulphur present in that part of peptone which is extractable by alcohol and also in the residue left after such extraction. This extraction with alcohol is the method given by Koch and Carr, Jour. Amer. Chem. Soc., (1909) 31, 1341, for separating lipoid sulphur compounds from protein sulphur compounds.

The apparatus used for the extraction was so designed that the extraction would take place at the boiling point of the solvent. It consisted of a very large test tube, 75 cm. long and 6 cm. in diameter, into the top of which was fitted by means of a one hole cork stopper another test tube, 50 cm. long and 4 cm. in diameter. Four holes had been bored in the bottom of this smaller test tube and one hole had been bored through its side about 5 cm. from the top. Into this tube just above the four holes in its bottom was fitted a perforated porcelain plate. On top of this plate was fitted tightly a circular piece of filter paper and on top of this filter paper was placed 200 grams of peptone. Into the top of this test tube containing the peptone there was fitted by means of a one hole cork stopper a Chamot-Soxhlet condenser to the mouth of which was connected a U tube containing calcium chloride to prevent the absorption of moisture

from the atmosphere by the alcohol used as the extracting agent. Five hundred cubic centimeters of 95 percent alcohol was placed in the larger tube, the apparatus connected together and clamped in place with the lower portion of the larger tube immersed in a paraffine bath. By heating this paraffine bath to from 140° to 145° C., the alcohol was boiled with sufficient vigor to drive its vapor up to the hole near the top of the inner tube, through which hole the vapor passed to the condenser where it was condensed and dripped down upon the peptone below. The extraction was carried on for 75 hours. At the end of each 15 hour period, the apparatus was taken apart, the peptone dried at a low heat, powdered and mixed and replaced in the smaller test tube, while the alcohol containing the extracted material was removed and 500 cc. of fresh alcohol substituted.

At the end of 75 hours, the alcohol was evaported from the combined extractions and the residue was dried at a low heat. Due to the gummy nature of this residue, it required several days to completely dry it.

The amount of material which had been extracted from the 200 grams of peptone originally employed was 50 grams. It seems hardly possible that so large a percent of peptone should be soluble in alcohol and the opinion is offered that most of the extracted material was really peptone which was dissolved by the water of the 95 percent alcohol used during the very long period of extraction.

TOTAL SULPHUR IN ALCOHOL EXTRACT.

After the material which had been extracted had been thoroughly air dried, 2 gram portions were analysed for total sulphur by the Liebig-Koch method.

TOTAL SULPHUR IN RESIDUE FROM ALCOHOL EXTRACTION.

Two gram portions of the thoroughly air dried residue left after extraction with alcohol were analysed for total sulphur by the Liebig-Koch method.

THE AVERAGE RESULTS FOR ALL OF THE FOREGOING METHODS APPEAR IN TABLE V.

TABLE V.

All determinations were made in triplicate.	Uncorrected per cent of sulphur in peptone.	Per cent of moisture in peptone **	Per cent of sulphur contributed by reagents.	Per cent of sulphur in peptone corrected for moisture and for sulphur contributed by reagents.
Liebig method (KOH, KNO_3 fusion)	.9282	6.19	.0040	.9902
Liebig method using less heat and more KNO_3	.9067	6.19	.0048	.9665
Osborne method (Na_2O_2 fusion)	.8888	6.19	.0000	.9479
A.O.A.C. method (Na_2O_2, Na_2CO_3 fusion)	.9122	6.19	.0056	.9664
Liebig-Koch method (HNO_3 digestion and KOH, KNO_3 fusion)	.9479	6.19	.0041	1.0061
HNO_3 digestion in closed system	.5005	6.19	.0012	.5323
HNO_3 digestion in open beakers	.4999	6.19	.0012	.5318
Distillate from HNO_3 digestion in closed system	.0041	6.19	.0043	.0000
Parr calorimeter	.9142	6.19	.0009	.9736
Schultz method for loosely bound sulphur	.3610	6.19	.0031	.3813
HNO_3, $KClO_3$ method for easily oxidized sulphur	.4899	6.19	.0007	.5194
HCl, $KClO_3$ method	.0723	6.19	.0010	.0761
Untreated peptone	.0004	6.19	.0000	.0004
Alcohol extract of peptone, Liebig-Koch method	.7013	12.30	.0041	.7950
Residue insoluble in alcohol, Liebig-Koch method	.9974	7.71	.0041	1.0763

**Determined by drying at 70° C. for 30 hours in vacuum over $CaCl_2$.

SUMMARY.

1. Of the methods tried, the Liebig-Koch method for tota sulphur gave the highest and most consistent results.

2. During the preliminary treatment with nitric acid in the Liebig-Koch method no sulphur is lost in volatile form.

3. The length of time beyond two hours during which nitric acid is allowed to act has no influence in the Liebig-Koch method.

4. Of the methods for determining a part only of the sulphur, the Schultz method for loosely bound sulphur and the digestion with nitric acid and potassium chlorate for easily oxidized sulphur gave the most consistent and most valuable results.

CHAPTER XII.

QUANTITATIVE DETERMINATIONS OF SULPHUR IN THE CULTURE MEDIUM FOR THE DETECTION OF THE BACTERIA PRODUCING HYDROGEN SULPHIDE.

By the use of the methods which had been found to give the best results in the determination of sulphur in peptone, presented in Chapter XI, a study was made of the total amount of sulphur in simple peptone culture media (see Chapter IX) broken down by the so-called putrefactive bacteria ; of the forms of sulphur most readily used by them ; and of the forms in which the sulphur existed after the action of the bacteria, whether as fixed sulphur, or as loosely bound sulphur, or as easily oxidized sulphur, or as a volatile sulphur compound such as hydrogen sulphide.

SULPHUR IN INSOLUBLE RESIDUE.

Determinations were first made of the total sulphur by the Liebig-Koch method (See Chapter XI) in that part of the peptone which is agglomerated and which appears as an insoluble residue when peptone is boiled with water, in order to ascertain whether this material contained such a large amount of sulphur that its removal by filtration might have a decided effect upon hydrogen sulphide production.

After corrections were made for the .0041 percent of sulphur contributed by the reagents, and for the 7.62 percent of moisture in the material used for analysis, it was found that the part of peptone coagulable in boiling water contained .9117 percent of sulphur, which is slightly less than the 1.0061 percent found in peptone itself. Hence, peptone solutions may be filtered without detriment to the medium.

PEPTONE AND SULPHUR USED UP BY BACTERIA IN FLASKS OF DIFFERENT SIZE.

In order to ascertain the amount of peptone used up by the bacteria and also the amount of sulphur used up in flasks of different size, when the simple peptone medium (see Chapter IX) was inoculated with artificial sewage (see Chapter II), 23 flasks of 100 cc. capacity were prepared by introducing into each, 10

cc. of 30. percent peptone solution and 5 cc. of 10. percent potassium chloride solution after which the flasks were plugged, sterilized and capped and then 85 cc. of artificial sewage which had been strained through sterile cloth into a sterile beaker was added to each flask. Also one flask of 2000 cc. capacity was prepared by introducing into it 200 cc. of 30. percent peptone solution and 100 cc. of 10. percent potassium chloride solution after which it was plugged, sterilized and capped and 1700 cc. of the same strained artificial sewage added to it as was used in the 100 cc. flasks.

Two thousand cubic centimeters of a mixture of 30. percent peptone solution, 10. percent potassium chloride solution, and artificial sewage in the same proportions as was used in both the small and large flasks above mentioned was prepared and sterilized to serve as a blank. The flasks containing the inoculated culture media were incubated for 48 hours at 38° C.

EFFECT OF POTASSIUM CHLORIDE AND OF SEWAGE ON SULPHUR DETERMINATIONS.

The grams per 100 cc. of total solids, the grams per 100 cc. of total sulphur by the Liebig-Koch method and the grams per 100 cc. of easily oxidized sulphur by the potassium chlorate-nitric acid method, were immediately determined in the blank and found to be 3.0690, .0309 and .0160 respectively. Computing to the basis of 3.0 grams per 100 cc. of total solids exclusive of potassium chloride, the amounts of total sulphur and of easily oxidized sulphur were .0302 and .0157 grams per 100 cc. respectively.

If the presence of potassium chloride and of artificial sewage has no influence on the sulphur determinations, then the grams of total sulphur per 100 cc. and of easily oxidized sulphur per 100 cc. divided by 3. and divided by 1.0110 (the specific gravity of a 3. percent peptone solution. (See Chapter II.) and multiplied by 100, should be very close to 1.0061 and .5194 respectively. Such computations actually give .9957 and .5176 and therefore, it is proved that the Liebig-Koch method for total sulphur and the potassium chlorate-nitric acid method for easily oxidized sulphur are as accurate for the culture media employed as they are for peptone itself.

FINAL REACTION OF CULTURES.

The contents of 3 of the 23 flasks of 100 cc. capacity were after 48 hours of incubation titrated at 20° C. with N/10 sodium hydroxide, using phenol phthalein as indicator, and it was found that the average reaction was 3.19 percent of normal acid (the average of 3.39, 3.02 and 3.17), showing that practically the same amount of decomposition had been produced by the bacteria as in the previous part of the work (see Chapter VIII) and that the two pieces of work are therefore comparable.

The cultures from 20 of the 100 cc. flasks were combined and filtered through a large webbed paper filter and the soluble material all washed through with distilled water ; then a hole was punched in the bottom of the filter and the insoluble material was washed with distilled water into a tared beaker.

The culture in the 2000 cc. flask was treated in like manner.

The soluble portions of the cultures were then evaporated to dryness to drive off all traces of volatile sulphur compounds, such as hydrogen sulphide ; dissolved in hot water and made up to 500 cc., when total solids exclusive of potassium chloride, total sulphur and easily oxidized sulphur were determined in measured portions.

The results are given in Table VI.

The insoluble portions of the cultures were evaporated to dryness on the water bath and then dried in a vacuum dessicator over calcium chloride at 75° C. and weighed. As the amount of material was so small, no attempt was made to determine the easily oxidized sulphur, all of the material being used in each case for the determination of total sulphur.

The results are given in Table VI.

DISCUSSION OF TABLE VI.

It should be noted that the ratio of total solids exclusive of potassium chloride to total sulphur is very nearly the same in the soluble and insoluble portions of both cultures, which would indicate a similarity of composition although it does not absolutely prove such a similarity. In other words, most of the insoluble material is probably peptone and therefore, it is of no advantage to separate the soluble and insoluble parts of the cultures by filtration for analysis and consequently such a separaton was not attempted in the subsequent work reported in this article.

TABLE VI.

All results are computed to the basis of 3.0 grams of total solids exclusive of KCl per 100 cc in the blank; .0302 grams being taken as the amount of total sulphur.	Grams per 100 cc. of total solids exclusive of KCl.	Grams per 100 cc. of unattacked sulphur.	Grams per 100 cc. of easily oxidized sulphur.	Per cent which easily oxidized sulphur is of unattacked sulphur.	Per cent of total solids, exclusive of KCl, destroyed.	Per cent of total sulphur converted into H_2S.	Per cent of easily oxidized sulphur converted into H_2S.
Peptone, potassium chloride blank	3.0000	.0302	.0157	51.99	-----	-----	-----
Cultures in 100 cc. flasks. Filtrate after 48 hours incubation	2.5885	.0145	.0103	71.03	13.72	51.99	34.39
Cultures in 2000 cc. flasks. Filtrate after 48 hours incubation	2.8850	.0180	.0106	58.89	3.83	40.40	32.48
Cultures in 100 cc. flasks. Sediment after 48 hours incubation	.0176	.0001	-----	-----	-----	-----	-----
Cultures in 2000 cc. flasks. Sediment after 48 hours incubation	.0121	.0001	-----	-----	-----	-----	-----
Cultures in 100 cc. flasks. Filtrate and sediment combined	2.6161	.0146	-----	-----	13.13	51.65	-----
Cultures in 2000 cc. flasks. Filtrate and sediment combined	2.8971	.0181	-----	-----	3.43	40 07	-----

EFFECT OF AMOUNT OF SURFACE EXPOSED.

The fact that the total solids exclusive of potassium chloride, total sulphur and easily oxidized sulphur were all markedly lower in the case of the cultures in the 100 cc. flask than in the case of the culture in the 2000 cc. flask was a fact which demanded explanation. A possible reason was that in the case of 100 cc. flasks there was a greater amount of surface exposed to a greater amount of air and that, in consequence, the bacteria were more energetic and therefore decomposed a larger amount of peptone. This idea is at variance with the belief that the organisms producing hydrogen sulphide are anaerobes.

In order to test out the validity of this explanation, two Fernbach culture flasks were prepared by introducing into each, 500 cc. from a mixture of 400 cc. of 30. percent peptone solution,

200 cc. of 10. percent potassium chloride solution and 3400 cc. of strained artificial sewage; two flasks of 500 cc. capacity were prepared by introducing into each, 500 cc. of the same mixture; ten flasks of 100 cc. capacity were prepared by introducing into each 100 cc. of the mixture, while the balance of the mixture was used as a blank.

The blank was immediately sterilized to await analysis while the inoculated flasks of various size and shape were placed in the 38° incubator. By means of a suction pump, air was passed through a wash bottle containing 5:1000 mercuric chloride solution to take out any hydrogen sulphide in the air, then through one of the Fernbach flasks and finally through a Fritz Friedrichs wash bottle containing 2.5 grams of cadmium chloride in water solution. The function of the cadmium chloride wash bottle was to catch and hold as cadmium sulphide all of the hydrogen sulphide which might be produced by the bacteria in the culture and swept over by the current of air. With the aid of screw clamps the speed of the air current was so regulated that one bubble per second passed through the wash bottles.

By means of the portable gas generator devised by A. W. Browne and M. J. Brown, Jour. Amer. Chem. Soc., 29, 859 (1907), carbon dioxide gas under constant pressure was generated from calcium carbonate and hydrochloric acid and passed first through a wash bottle containing distilled water, then through the other Fernbach flask and finally through a Fritz Friedrichs wash bottle containing 2.5 grams of cadmium chloride in water solution, wherein any hydrogen sulphide, generated by the bacteria in the culture would be precipitated as cadmium sulphide. The carbon dioxide generator was so regulated that one bubble of gas per second passed through the wash bottle.

The Fernbach flasks through which air and carbon dioxide respectively were being passed and the other flasks through which no gas was being passed but which were being allowed to incubate in the usual manner, were all left in the 38° incubator for 48 hours. They were then sterilized by heat. Without filtering off the insoluble material, this having been proved to be unnecessary in the previous set of inoculations, the blank and the various cultures were analysed for total solids, for total sulphur, for easily oxidized sulphur, for loosely bound sulphur by the Schultz method, and for sulphur as hydrogen sulphide. For making these determinations, except that of sulphur as hydrogen sulphide, the blank and the various cultures were evaporated separately to small volumes, then each was made up to 250 cc. in a measuring flask and measured portions taken for analysis.

For the determination of sulphur as hydrogen sulphide, the contents of the cadmium chloride wash bottles were acidified with strong hydrochloric acid, an excess of N/10 iodine added and the excess titrated with N/10 sodium thiosulphate.

The results are given in Table VII.

TABLE VII.

All results are computed to the basis of 3.0 grams of total solids exclusive of KCl per 100 cc. in the blank; .0302 grams being taken as the amount of total sulphur.

	Grams per 100 cc. of total solids exclusive of KCl.	Grams per 100 cc. of unattacked sulphur.	Grams per 100 cc. of easily oxidized sulphur.	Grams per 100 cc. of loosely bound sulphur.	Grams per 100 cc. of sulphur as H_2S.	Percent which easily oxidized sulphur is of unattacked sulphur.	Percent which loosely bound sulphur is of unattacked sulphur.	Sum of unattacked sulphur plus sulphur as H_2S.	Percent of sulphur accounted for.	Percent of total solids, exclusive of KCl, destroyed.	Percent of total sulphur converted into H_2S.	Percent of easily oxidized sulphur converted into H_2S.	Percent of loosely bound sulphur converted into H_2S.
Peptone, potassium chloride blank	3.0000	.0302	.0157	.0078	-----	51.99	25.83	.0302	100.00	-----	-----	-----	-----
Culture in Fernbach. Air passed over. Incubated 48 hours	2.6520	.0200	.0090	.0041	.0011	45.00	20.50	.0211	69.87	11.60	33.77	42.68	47.45
Cultures in 100 cc. flasks. Incubated 48 hours	2.7209	.0222	.0105	.0049	-----	47.30	22.07	-----	-----	9.30	26.49	33.12	37.18
Cultures in 500 cc. flasks. Incubated 48 hours	2.7872	.0244	.0110	.0055	-----	45.08	22.54	-----	-----	7.09	19.21	29.93	29.49
Culture in Fernbach. Carbon dioxide passed over. Incubated 48 hours	2.8899	.0249	.0096	.0066	.0012	38.55	26.51	.0261	86.42	3.67	17.55	38.85	15.38

DISCUSSION OF TABLE VII.

The amounts of total solids, exclusive of potassium chloride, remaining in the media after incubation proved that the bacterial action was most energetic in the Fernbach through which air was passed ; next in the cultures having the greatest surface exposed *i.e.* in the 100 cc. flasks ; next in the cultures in the 500 cc. flasks ; and was least energetic in the Fernbach flask through which carbon dioxide was passed ; while the amounts of total sulphur remaining, the difference between which and the total sulphur of the blank is a measure of the volatile sulphur compounds produced by the bacteria, proved that the production of volatile sulphur compounds by the bacteria was most energetic in the Fernbach through which air was passed ; next in the cultures in 100 cc. flasks ; next in the cultures in 500 cc. flasks ; and was least energetic in the Fernbach flask through which carbon dioxide was passed. Therefore, the orders for general bacterial activity and for hydrogen sulphide production were identical, and in the case of the flora with which this work was done, at least, the organisms producing hydrogen sulphide in largest amounts were aerobes and not anaerobes and for the maximum production of hydrogen sulphide, broad, low flasks giving the maximum surface of culture exposed to air should be used. The form of flask illustrated in Cut 1 at the end of the article could be improved by making it lower and broader.

DETERMINATIONS OF HYDROGEN SULPHIDE.

The fact that the amounts of sulphur as hydrogen sulphide found by the method employed were far from being the differences between the total sulphur of the blank and the total sulphurs of the cultures in which sulphur as hydrogen sulphide was determined, demanded attention, as it indicated that the method which had been employed for determining hydrogen sulphide was faulty and that the cadmium chloride had failed to stop all of the hydrogen sulphide evolved. It was therefore decided to try an iodine solution instead of the cadmium chloride solution in the wash bottle.

For this purpose, air was drawn by suction for 48 hours, first through a wash bottle containing lead acetate solution to remove

any gaseous sulphur compounds, then through a Fernbach flask containing 500 cc. of the culture medium, artificial sewage mixture already described, said flask being in the 38° incubator, then through what had been calculated to be an excess of N/10 iodine solution in a Fritz Friedrichs wash bottle and finally through a N/10 sodium thiosulphate solution in a wash bottle. The sodium thiosulphate was used to stop and hold any iodine which might be volatilized.

During the period of incubation, the iodine solution was found to fade rapidly, indicating that larger amounts of hydrogen sulphide than had been expected were being produced, and consequently, small amounts of normal iodine solution were, from time to time, added to the iodine solution in the wash bottle. After 48 hours of incubation, the iodine solution and the sodium thiosulphate solution were mixed together and the amount of iodine which had been used up, determined by titration.

It was found that an amount of iodine equivalent to an amount of hydrogen sulphide which would be produced by nearly all of the sulphur of the medium being transformed to hydrogen sulphide, had been reduced. As determinations of total sulphur showed that only about 25 percent of the total sulphur had disappeared from the medium the results were evidently impossible. The explanation which is offered is that volatile, unsaturated organic compounds like indol, $C_6H_4\begin{smallmatrix}/\,NH\,\backslash\\ \backslash\,CH\,=\end{smallmatrix}CH$ and skatol, $C_6H_4\begin{smallmatrix}/\,NH\,\backslash\\ \backslash\,C(CH_3)=\end{smallmatrix}CH$ were liberated from the medium by the action of the bacteria and that these reduced the iodine solution.

The use of iodine solution having been proved to be of no value, it was decided to try solutions of cadmium chloride containing potassium chloride to prevent colloidal suspension of cadmium sulphide, of lead acetate containing potassium nitrate to prevent colloidal suspension of lead sulphide, of sodium peroxide and of potassium hydroxide, determining the total sulphur retained as sulphide by the Liebig-Koch method and not by liberating with hydrochloric acid and titrating with iodine and sodium thiosulphate solutions as had been done when cadmium chloride was before used.

The Fernbach flasks containing the usual mixture of culture medium and artificial sewage and the Fritz Friederichs wash bottles containing the various solutions were connected in parallel to the same suction.

The blank was immediately sterilized to await analysis and the Fernbachs were incubated at 38° for 72 hours (allowed to incubate 72 hours because the end of 48 hour period came on Sunday), air being sucked through the Fernbach flasks and their wash bottles at as uniform a rate as possible.

After 72 hours, each Fernbach was heated to boiling on a water bath for 30 minutes while air was sucked through it and its respective wash bottles. This was to drive out all hydrogen sulphide held in solution in the cultures. The solution from each Fernbach was then made up to 500 cc. (only a few cc. had been lost by evaporation in each case), and total solids, total sulphur and sulphur as non-volatile sulphides determined in measured portions of these cultures and of the blank. The determination of sulphur as non-volatile sulphides which has not been yet described was accomplished by placing 300 cc. of each culture in a distilling flask and adding 25 cc. of concentrated hydrochloric acid. A Vigreux tube was attached and the solution was then distilled for 30 minutes under reduced pressure into sodium peroxide solution (5 : 60) contained in a wash bottle, the purpose being to decompose the sulphides and to distil the hydrogen sulphide formed into the sodium peroxide where it would be held as sodium sulphide, in which form the sulphur was determined by the Liebig-Koch method.

The results are given in Table VIII.

DISCUSSION OF TABLE VIII.

As the sum of the total sulphur remaining in the medium plus the sulphur found as hydrogen sulphide in the wash bottles proved to be .0294 grams per 100 cc. when cadmium chloride was used, .0245 when lead acetate was used, .0277 when sodium peroxide was used and .0302 when potassium hydroxide was used, while .0302 grams of total sulphur per 100 cc., on the basis of 3. grams of total solids exclusive of potassium chloride per 100 cc., had been found as the result of a number of determinations to be the amount present in the blanks, the indications were that potassium hydroxide stopped and held all of the volatile sulphur compounds evolved while the other substances failed to do so, although cadmium chloride was almost as efficient.

TABLE VIII.

All results are computed to the basis of 3.0 grams of total solids exclusive of KCl per 100 cc. in the blank; .0302 grams being taken as the amount of total sulphur.

	Grams per 100 cc. of total solids exclusive of KCl.	Grams per 100 cc. of unattacked sulphur.	Grams per 100 cc. of sulphur as non-volatile sulphide.	Grams per 100 cc. of sulphur as H_2S.	Sum of unattacked sulphur plus sulphur as H_2S.	Percent of sulphur accounted for.	Per cent of total solids, exclusive of KCl, destroyed.	Percent of total sulphur converted into H_2S.	Percent of total sulphur converted into non-volatile sulphide.
Peptone, potassium chloride blank	3.0000	.0300	.0003	-----	.0300	99.34	-----	-----	----
Culture in Fernbach. Air passed over. Incubated 72 hours. Potassium hydroxide in wash bottle	2.4915	.0148	.0003	.0154	.0302	100.00	16.95	51.00	.00
Culture in Fernbach. Air passed over. Incubated 72 hours. Cadmium chloride in wash bottle	2.5535	.0154	.0009	.0140	.0294	97.35	14.88	49.01	1.99
Culture in Fernbach. Air passed over. Incubated 72 hours. Sodium peroxide in wash bottle	2.5118	.0133	.0009	.0144	.0277	91.72	16.27	55.96	1.99
Culture in Fernbach. Air passed over. Incubated 72 hours. Lead acetate in wash bottle	2.4023	.0133	.0010	.0112	.0245	81.13	19.92	55.96	2.32

DETERMINATIONS OF HYDROGEN SULPHIDE WITH SHORTER INCUBATION.

To eliminate the possibility of analytical errors having crept into this important determination, another exactly similar set of inoculations and sulphur determinations (with the exception that the cultures were incubated 48 hours instead of 72 hours) was made.

The results are given in Table IX.

DISCUSSION OF TABLE IX.

In this second set of determinations the results in all ways confirmed those of the previous work. The sum of the total sulphur remaining in the medium plus the sulphur found as hydrogen sulphide in the wash bottles proved to be .0304 grams per 100 cc. when cadmium chloride was used, .0243 when lead acetate was used, .0302 when sodium peroxide was used and .0299 when potassium hydroxide was used, showing that cadmium chloride, sodium peroxide and potassium hydroxide were all efficient reagents to use, while lead acetate was not. In subsequent work, the three reagents named were all employed although potassium hydroxide is preferable because the precipitate of cadmium sulphide tends to stick to the sides of the wash bottles from which it must be dissolved by the potassium chlorate-nitric acid reagent with a possibility of sulphur being lost as hydrogen sulphide before it can be oxidized by the reagent, while there is a bad tendency toward spattering in the alkaline fusion when sodium peroxide is present, with possibility of loss. Neither of these difficulties is encountered when potassium hydroxide is used to catch and hold the volatile sulphur compounds.

It was very gratifying to have the sums of the volatile and non-volatile sulphur compounds in the different cultures approximate so closely to the amount of total sulphur found in the blanks, as it showed that the analytical methods which had been adopted are capable of giving results which are surprisingly accurate when one considers the large number of operations, transferences and filtrations incident to the methods.

TABLE IX.

All results are computed to the basis of 3.0 grams of total solids exclusive of KCl per 100 cc. in the blank; .0302 grams being taken as the amount of total sulphur.

	Grams per 100 cc. of total solids exclusive of KCl.	Grams per 100 cc. of unattacked sulphur.	Grams per 100 cc. of sulphur as non-volatile sulphide.	Grams per 100 cc. of sulphur as H_2S.	Sum of unattacked sulphur plus sulphur as H_2S.	Percent of sulphur accounted for.	Percent of total solids, exclusive of KCl, destroyed.	Percent of total sulphur converted into H_2S.	Percent of total sulphur converted into non-volatile sulphide.
Peptone, potassium chloride blank	3.0000	.0301	.0002	-----	.0301	99.67	----	-----	-----
Culture in Fernbach. Air passed over. Incubated 48 hours. Cadmium chloride in wash bottle	2.8136	.0210	.0008	.0094	.0304	100.66	6.21	30.46	1.65
Culture in Fernbach. Air passed over. Incubated 48 hours. Sodium peroxide in wash bottle	2.9064	.0222	.0014	.0080	.0302	100.00	3.12	26.49	3.64
Culture in Fernbach. Air passed over. Incubated 48 hours. Potassium hydroxide in wash bottle	2.7296	.0189	.0007	.0110	.0299	99.01	9.01	37.42	1.32
Culture in Fernbach. Air passed over. Incubated 48 hours. Lead acetate in wash bottle	2.8055	.0209	.0013	.0034	.0243	80.46	6.48	30.79	3.31

MERCAPTANS.

If mercaptans as well as hydrogen sulphide are formed by the action of the bacteria of sewage, then these mercaptans which boil at temperatures below that of the incubator would pass over into the wash bottles, there to be held as metallic derivatives just as hydrogen sulphide would be held, and subsequently included in the determination of the total sulphur of the material in the wash bottles by the Liebig-Koch method. Hence, they would be included under what has been designated sulphur as hydrogen sulphide. The amounts of mercaptans formed, if any, would undoubtedly be very small and consequently no attempt was made to separate and determine them.

SULPHUR IN ALCOHOL EXTRACT OF PEPTONE AND IN RESIDUE FROM ALCOHOL EXTRACTION.

In order to ascertain whether the bacteria present in artificial sewage produce hydrogen sulphide more energetically or less energetically from those portions of peptone which are soluble in alcohol (containing lipoid sulphur) and insoluble in alcohol (containing protein sulphur) than they do from peptone itself, culture media were made, in one of which, the part of peptone insoluble in alcohol was substituted for peptone, and in the other of which, the part of peptone soluble in alcohol was substituted for peptone. The portions of peptone soluble and insoluble in in alcohol which were employed, were obtained in the course of the work described in Chapter XI.

HYDROGEN SULPHIDE PRODUCTION FROM THE PART OF PEPTONE INSOLUBLE IN ALCOHOL.

Two Fernbach culture flasks were prepared by introducing into each, 500 cc. from a mixture of 150 cc. of a 30. percent solution of the part of peptone insoluble in alcohol, 75 cc. of 10. percent potassium chloride solution and 1275 cc. of strained artificial sewage. The remaining 500 cc. was immediately sterilized and kept as a control to be analysed. One of the Fernbachs was attached to a wash bottle containing sodium peroxide solution (15:225) and the other was attached to a wash bottle containing potassium hydroxide solution (20:225) arranged as previously described. One Fernbach flask to act as

a check, was prepared by introducing into it 500 cc. from a mixture of 100 cc. of 30. percent peptone solution, 50 cc. of 10. percent potassium chloride solution and 850 cc. of strained artificial sewage. The remaining 500 cc. was immediately sterilized and kept as a blank to be analysed. The Fernbach flask was attached to a wash bottle containing sodium peroxide solution (15:225).

The three Fernbachs were incubated for 48 hours at 38°, air in each case, being sucked through a test tube containing the same solution as the respective wash bottle, then through the Fernbach flask, and finally through the wash bottle at as uniform a rate as possible.

After 48 hours of incubation, each Fernbach was heated to boiling on a water bath for 30 minutes while air was sucked through it and its respective wash bottle. The solution from each Fernbach was then made up to 500 cc. (only 1 cc. to 2 cc. had been lost by evaporation in each case) and total solids, total sulphur, sulphur as non-volatile sulphide and sulphur as hydrogen sulphide were determined in measured portions. In the determinations of sulphur as non-volatile sulphide, the distillates were caught in 12. percent potassium hydroxide solution.

The results of the analysis are given in Table X.

DISCUSSION OF TABLE X.

The fact to be especially noted is that in both of the Fernbachs containing the medium made from the part of peptone insoluble in alcohol, much less hydrogen sulphide was produced than had been produced in media made from peptone itself. That this was not due to a less energetic bacterial flora in the artificial sewage than had been used in previous work, is proved by the fact that in the check Fernbach made up with the regular peptone medium and with the same sewage, the amounts of total solids exclusive of potassium chloride, total sulphur, sulphur as non-volatile sulphide and sulphur as hydrogen sulphide ; computed to the basis of 3. grams of total solids exclusive of potassium chloride per 100 cc. in the blank ; were 2.7836, .0242, .0006 and .0059 grams per 100 cc. respectively, more than twice as much hydrogen sulphide having been produced as in either of the cultures containing the part of peptone insoluble in alcohol.

TABLE X.

All results are computed to the basis of 3.0 grams of total solids exclusive of KCl in the 100 cc. blanks.

	Grams per 100 cc. of total solids exclusive of KCl.	Grams per 100 cc. of unattacked sulphur.	Grams per 100 cc. of sulphur as non-volatile sulphide.	Grams per 100 cc. of sulphur as H_2S.	Sum of unattacked sulphur plus sulphur as H_2S.	Percent of sulphur accounted for.	Percent of total solids, exclusive of KCl, destroyed.	Percent of total sulphur converted into H_2S.	Percent of total sulphur converted into non-volatile sulphide.
Peptone, potassium chloride blank	3.0000	.0303	.0003	-----	.0303	100.33	----	-----	----
Culture in Fernbach. Air passed over. Incubated 48 hours. Sodium peroxide in wash bottle	2.7836	.0242	.0006	.0059	.0301	99.67	7.21	19.87	.99
Part of peptone insoluble in alcohol, potassium chloride control	3.0000	.0324	.0005	----	.0324	100.00	----	-----	----
Culture in Fernbach. Air passed over. Incubated 48 hours. Sodium peroxide in wash bottle	2.8055	.0299	.0009	.0024	.0323	99.69	6.48	7.72	1.23
Culture in Fernbach. Air passed over. Incubated 48 hours. Potassium hydroxide in wash bottle	2.8986	.0303	.0010	.0009	.0312	96.30	3.38	6.48	1.54

HYDROGEN SULPHIDE PRODUCTION FROM PART OF PEPTONE SOLUBLE IN ALCOHOL.

A set of inoculations was made using a culture medium made from the part of peptone soluble in alcohol.

Two Fernbach culture flasks were prepared by introducing into each, 500 cc. from a mixture of 150 cc. of a 30. percent aqueous solution of the part of peptone soluble in alcohol, 75 cc. of 10. percent potassium chloride solution and 1275 cc. of strained artificial sewage. The remaining 500 cc. was immediately sterilized and kept as a control to be analyzed. One of the Fernbachs was attached to a wash bottle containing cadmium chloride solution (10 : 225) and the other was attached to a wash bottle containing potassium hydroxide solution (20 : 225) arranged as previously described. Two Fernbach culture flasks to act as checks were also prepared by introducing into each, 500 cc. from a mixture of 150 cc. of 30. percent peptone solution, 75 cc. of 10. percent potassium chloride solution and 1275 cc. of strained artificial sewage. The remaining 500 cc. was immediately sterilized and kept as a blank to be analyzed. One of the check Fernbachs was attached to a wash bottle containing cadmium chloride solution (10:225) and the other was attached to a wash bottle containing potassium hydroxide solution (20 : 225) arranged as previously described.

The four Fernbachs were incubated for 48 hours at 38°, air in each case being sucked through a test tube containing the same solution as the respective wash bottle then through the Fernbach flask and finally through the wash bottle at as uniform a rate as possible.

After 48 hours of incubation, each Fernbach was heated to boiling on a water bath for 30 minutes while air was sucked through it and its respective wash bottle. The solution from each Fernbach was then made up to 500 cc. (only about 3 cc. had been lost by evaporation in each case) and the total solids, total sulphur, sulphur as non-volatile sulphides and sulphur as hydrogen sulphide were determined in measured portions.

The results of analysis are given in Table XI.

DISCUSSION OF TABLE XI.

The fact to be especially noted is that in both of the Fernbachs containing the medium made from the part of peptone soluble in alcohol, much less sulphur-containing material was broken down and very much less hydrogen sulphide was produced than had been produced in media made from the part of peptone insoluble in alcohol which in turn had given smaller amounts than

TABLE XI.

All results are computed to the basis of 3.0 grams of total solids exclusive of KCl in the 100 cc. blanks.

	Grams per 100 cc. of total solids exclusive of KCl.	Grams per 100 cc. of unattacked sulphur.	Grams per 100 cc of sulphur as non-volatile sulphide.	Grams per 100 cc. of sulphur as H_2S.	Sum of unattacked sulphur plus sulphur as H_2S.	Percent of sulphur accounted for.	Per cent of total solids, exclusive of KCl, destroyed.	Per cent of total sulphur converted into H_2S.	Percent of total sulphur converted into non-volatile sulphide.
Peptone, potassium chloride blank	3.0000	.0302	.0002	-----	.0302	100.00	----	-----	----
Culture in Fernbach. Air passed over. Incubated 48 hours. Potassium hydroxide in wash bottle	2.8430	.0242	.0006	.0064	.0306	101.32	5.23	19.87	.99
Culture in Fernbach. Air passed over. Incubated 48 hours. Cadmium chloride in wash bottle	2.8092	.0224	.0013	.0072	.0296	98.01	6.36	25.83	3.31
Part of peptone soluble in alcohol, potassium chloride control	3.0000	.0228	.0002	-----	.0228	100.00	----	-----	----
Culture in Fernbach. Air passed over. Incubated 48 hours. Potassium hydroxide in wash bottle.	2.8825	.0225	.0003	.0003	.0228	100.00	3.92	1.01	.44
Culture in Fernbach. Air passed over. Incubated 48 hours. Cadmium chloride in wash bottle	2.9220	.0224	.0003	.0001	.0225	98.68	2.60	1.75	.44

had been found in media made from peptone itself. That this was not due to a less energetic bacterial flora in the artificial sewage than had been present in previous tests is proven by the fact that in the check Fernbach flasks made up with the regular peptone medium and with the same sewage, the amounts of total solids exclusive of potassium chloride, of total sulphur, of sulphur as non-volatile sulphide and of sulphur as hydrogen sulphide ; computed to the basis of 3. grams of total solids exclusive of potassium chloride per 100 cc. in the blank ; were in the cases where cadmium chloride and potassium hydroxide respectively had been used in the wash bottles, 2.8092, .0224, .0013, .0072 grams per 100 cc.; and 2.8430, .0242, .0006, .0064 grams per 100 cc.

Hence, it is evident that a medium made with peptone as the sulphur-containing material is a better one to use for the detection of the bacteria producing hydrogen sulphide than are media made with the part of peptone soluble in alcohol or with the part of peptone insoluble in alcohol.

CALLIBRATION OF BLACKENING OF LEAD ACETATE PAPERS IN NECKS OF CULTURE FLASKS.

As it had been repeatedly demonstrated in this piece of work that the amount of hydrogen sulphide produced might be measured by the difference between the amount of total sulphur present in the culture medium before incubation and the amount of total sulphur remaining after incubation, provided that the culture after incubation was boiled to drive out all gas held in solution, a series of determinations was made to ascertain the actual amounts of hydrogen sulphide corresponding to different amounts of blackening of lead acetate paper when the 100 cc. flasks illustrated in Cut 1 were used and when strips of lead acetate paper 2 mm. in width, were placed in the tubular part of the caps.

For this purpose, a mixture of 250 cc. of 30. percent peptone solution, 125 cc. of 10. percent potassium chloride solution and 2125 cc. of strained artificial sewage was made and 100 cc. portions of this mixture were introduced into 20 culture flasks each of which had been provided with a strip of lead acetate paper and a tin foil cap in the usual manner. The remaining 500 cc. was immediately sterilized and held as a blank for analysis.

The flasks were placed in the 38° incubator and left until the lead acetate papers began to blacken. As soon as a flask was

found in which the paper was blackened for 1 mm., it was taken out and boiled to sterilize it and to drive off any hydrogen sulphide held in solution and the total sulphur remaining in the medium was determined by the Liebig-Koch method. As soon as a flask was found in which the paper had blackened for 2 mm., it was removed from the incubator and treated in like manner. In this way, flasks in which the paper was blackened for 3 mm., 4 mm., 5 mm. etc., up to 20 mm., were removed and the contents analyzed for total sulphur. The blank was also analyzed for total sulphur.

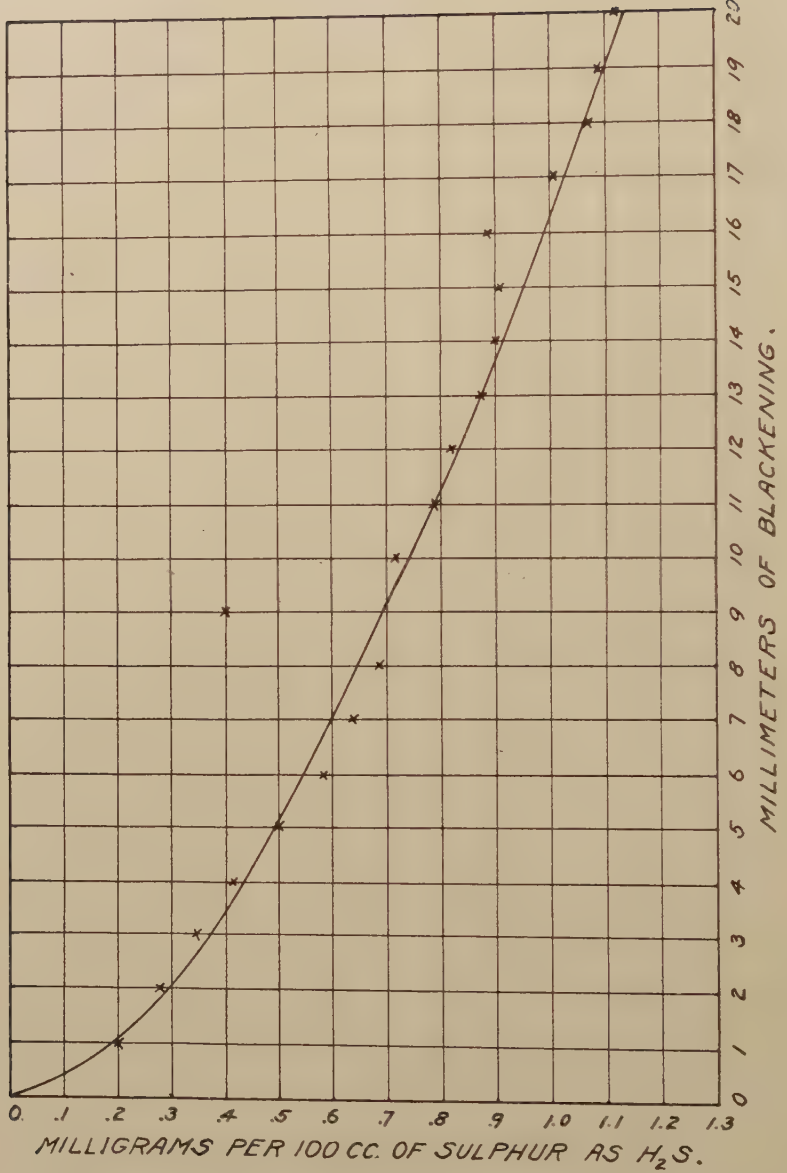
With the single exception of the culture in which the lead acetate paper was blackened for 9 mm. (the abnormal results of which can be explained only on the basis of experimental errors) the amounts of sulphur as hydrogen sulphide show a gradual increase with the increasing blackening of the lead acetate paper.

The results are given in Table XII, and are also represented graphically in the curve which follows the table.

TABLE XII.

All results are computed to the basis of 3.0 grams of total solids exclusive of KCl in the 100 cc. blank.

Millimeters blackened of lead acetate paper.	Grams per 100 cc. of barium sulphate.	Grams per 100 cc. of sulphur remaining in the culture.	Grams per 100 cc. of sulphur as hydrogen sulphide.
0	.2198	.0302	----
1	.2181	.0300	.0002
2	.2177	.0299	.0003
3	.2175	.0299	.0003
4	.2170	.0298	.0004
5	.2161	.0297	.0005
6	.2155	.0296	.0006
7	.2153	.0296	.0006
8	.2147	.0295	.0007
9	.2170	.0298	.0004
10	.2143	.0295	.0007
11	.2139	.0294	.0008
12	.2138	.0294	.0008
13	.2128	.0292	.0010
14	.2124	.0292	.0010
15	.2123	.0292	.0010
16	.2125	.0292	.0010
17	.2119	.0291	.0011
18	.2113	.0290	.0012
19	.2112	.0290	.0012
20	.2111	.0290	.0012


0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
MILLIMETERS OF BLACKENING.
0. .1 .2 .3 .4 .5 .6 .7 .8 .9 1.0 1.1 1.2 1.3
MILLIGRAMS PER 100 CC. OF SULPHUR AS H_2S.

SUMMARY.

1. The percent of sulphur present in the very small part of peptone which is insoluble in water is practically the same as in peptone itself. This explains why, in testing for bacteria which produce hydrogen sulphide, it makes no difference whether the medium is filtered or not.

2. The Liebig-Koch method for total sulphur and the potassium chlorate-nitric acid method for easily oxidized sulphur are as accurate for culture media made from peptone and potassium chloride as they are for peptone itself.

3. The ratio of total solids exclusive of potassium chloride to total sulphur is very nearly the same in the soluble and insoluble portions of cultures which have been inoculated with artificial sewage, containing bacteria which produce hydrogen sulphide, and incubated at 38° for 48 hours, and hence the indications are that the insoluble material is mostly peptone.

4. More material is broken down by the bacteria and more hydrogen sulphide is produced in flasks having much surface of the culture exposed to air than in flasks having comparatively little surface exposed.

5. It is noteworthy that about 50. percent more total sulphur was converted into hydrogen sulphide when sterile air was passed over the cultures as when they were exposed to quiescent air; and about 100. percent more total sulphur was, under otherwise identical conditions, converted into hydrogen sulphide when sterile air was passed over the cultures as when sterile carbon dioxide was passed over them. Therefore, in the case of the flora which was used, at least, the organisms were most active and produced the most hydrogen sulphide when supplied most freely with air. This fact would appear to be of great importance in connection with work directed toward the elimination of odors arising from septic tanks for sewage disposal.

6. From 25. percent to 30. percent of the total sulphur was converted into hydrogen sulphide when cultures were incubated 48 hours with sterile air passing over the culture, and from 50. percent to 60. percent was thus converted when incubated 72 hours.

7. The volumetric iodine method for the determination of hydrogen sulphide is not available in the analysis of the gases

given off by bacteria inoculated into the simple peptone medium, because of the interference of volatile unsaturated organic compounds.

8. Solutions of cadmium chloride containing potassium chloride to prevent colloidal suspension, of sodium peroxide and of potassium hydroxide are available for use in wash bottles through which the gases given off by bacteria may be drawn for the purpose of catching and holding the hydrogen sulphide as metallic sulphide in which form the sulphur may be determined by the Liebig-Koch method. The use of potassium hydroxide is to be preferred.

9. The sum of the sulphur remaining in the cultures after incubation plus the sulphur in gaseous form, which is practically all present as hydrogen sulphide, equals the total sulphur present in the cultures before incubation, when the determinations are made by the methods adopted in this investigation.

10. In media made from the part of peptone soluble in alcohol much less sulphur-containing material was broken down and very much less hydrogen sulphide was produced than was found in the media made from the part of peptone insoluble in alcohol, the ratios being about 3 : 5 and 1 : 6 ; while in both of these media, very much less sulphur-containing material was broken down and very much less hydrogen sulphide was produced than in the simple peptone medium, the ratios being respectively about 1 : 2 and 1 : 20 in the soluble part of peptone, and 2 : 3 and 1 : 4 in the insoluble part of peptone.

11. As shown by the percent of total solids exclusive of potassium chloride destroyed, and by the percent of total sulphur converted into hydrogen sulphide, a larger percent of sulphur-containing material than of total peptone was broken down, the ratio being about 3:1. The fact that the bodies of the bacteria were included in the total solids exclusive of potassium chloride, would not materially influence the ratio.

12. A larger percent of easily oxidized sulphur than of total sulphur was converted into hydrogen sulphide by the bacteria, the ratio being about 4:3. It is evident that if a synthetic medium is to be prepared for the detection of the bacteria producing hydrogen sulphide, work upon which is herewith promised, that the sulphur should be introduced in an easily oxidized form.

13. A larger percent of loosely bound sulphur than of total sulphur was converted into hydrogen sulphide by the bacteria, the ratio being about 3:2.

14. A very slightly greater percent of loosely bound sulphur than of easily oxidized sulphur was converted into hydrogen sulphide by the bacteria, the ratio being about 10:9. There was about twice as much easily oxidized sulphur as there was loosely bound sulphur, both before and after the bacteria had acted upon the medium.

15. It is possible with a fair degree of accuracy to determine the equivalent of hydrogen sulphide for each millimeter of blackening of the lead acetate paper strips in the caps of the special flasks.

CHAPTER XIII.

COMPARISON OF THE RAPIDITY OF HYDROGEN SULPHIDE PRODUCTION AND OF THE AMOUNTS PRODUCED WHEN ARTIFICIAL SEWAGES MADE FROM HUMAN FECES AND FROM THE FECES OF VARIOUS ANIMALS WERE USED.

In order to ascertain whether the feces of the domestic animals contribute to sewage, bacteria which produce hydrogen sulphide and in what relative amounts, a comparison was made using different artificial sewages.

A number of the flasks illustrated in Cut 1 were prepared by introducing into each, 100 cc. of a solution containing 30. percent of peptone and 5. percent of potassium chloride, after which the flasked media were sterilized in the autoclave at three fourths of an atmosphere, lead acetate papers were introduced and the flasks capped with tin foil.

Another group of flasks of the same kind were prepared in exactly similar manner except that 80 cc. of Cornell University tap water was also introduced into each before sterilization.

Artificial sewages were prepared by introducing into 1000 cc. portions of Cornell University tap water one loop full (.02 grams) of human feces, one loop full of feces from Cow I, one loop full of feces from Cow II, one loop full of feces from Calf I, one loop full of feces from Horse I, one loop full of feces from Horse II, one loop full of feces from Pig I, one loop full of feces from Pig II, and one loop full of feces from Sheep I respectively.

Ninety cubic centimeters of each of the sewages was introduced respectively into each of two of the culture flasks containing the small volume of medium, and 10 cc. of each of the sewages was introduced respectively into each of two of the culture flasks containing the large volume of medium and then all of the 36 flasks were placed in the 38° incubator.

The amounts of hydrogen sulphide produced may be most easily presented in tabular form and are given in Table XIII.

TABLE XIII.

Amount of sewage inoculated, and kind.	Millimeters blackened of lead acetate papers in				
	18 hrs.	21 hrs.	24 hrs.	36 hrs.	48 hrs.
10 cc. human	0	0	0	4	12
90 cc. human	0	1	5	all	all
10 cc. cow I.	0	1	2	10	all
90 cc. cow I.	0	0	0	8	all
10 cc. cow II.	0	0	0	12	all
90 cc. cow II.	0	1	4	all	all
10 cc. calf I.	0	1	4	all	all
90 cc. calf I.	2	5	8	all	all
10 cc. horse I.	0	0	0	14	all
90 cc. horse I.	0	0	1	6	all
10 cc. horse II.	0	0	0	14	all
90 cc. horse II.	0	0	1	5	20
10 cc. pig I.	0	0	0	17	all
90 cc. pig I.	0	0	1	15	all
10 cc. pig II.	0	1	2	16	all
90 cc. pig II.	0	1	4	all	all
10 cc. sheep I.	0	0	1	8	all
90 cc. sheep I.	0	0	0	6	20

DISCUSSION OF TABLE XIII.

It should be noted that hydrogen sulphide had been produced in 24 hours by the organisms from the feces of all of the animals tried, namely, man, cow, horse, pig and sheep and that in 36 hours large amounts of hydrogen sulphide had been produced in every case while the solutions were all greenish in color and very turbid, much gas had formed on them and dark green floating cakes and sediment had been formed.

SUMMARY.

The feces of the domestic animals contain bacteria which are capable of producing hydrogen sulphide from the simple peptone medium as rapidly and in as large amounts, as is the case with the bacteria from human feces.

CHAPTER XIV.

TESTS FOR HYDROGEN SULPHIDE PRODUCTION MADE WITH DIFFERENT STRAINS OF THE COLON ORGANISM ISOLATED FROM HUMAN FECES.

For the purpose, 68 flasks of 100 cc. capacity were prepared by introducing into each one, 10 cc. of a solution containing 30. percent of peptone and 5. percent of potassium chloride, and 90 cc. of Cornell University tap water. This mixture was quite turbid but when sterilized at three fourths of an atmosphere in an autoclave, the solution in each flask became perfectly clear with a slight sediment at the bottom due to the agglomeration of the colloidal peptone which had made the solution turbid before sterilization.

As both dextrose peptone and lactose neutral red cultures of 34 different strains of the colon organism were available, a flask of media was inoculated with a four millimeter loop full of each of the cultures of each of the strains, making 68 in all. The flasks were then provided with strips of lead acetate paper in the tubular part of the caps, were capped tightly with tin foil, and placed in the 38° incubator and observed at intervals after 18 hours.

In Table XIV are presented the results of these inoculations, the number of millimeters which the lead acetate paper was blackened on first observing hydrogen sulphide production in each culture being given. For the sake of clearness, the increased blackening of the papers is not recorded.

After 8 days, no more evidence of any production of hydrogen sulphide was noted even though the cultures were left in the incubator for 30 days and a "O" has been placed in the 8 day column against each culture which failed to produce any hydrogen sulphide.

There were in all, 52 of the cultures which produced hydrogen sulphide in from 1 to 8 days, while 16 failed to do so. Of the 34 strains, 21 produced hydrogen sulphide from both the dextrose peptone and from the lactose neutral red cultures, 10 produced hydrogen sulphide when inoculated from one culture but not from the other, while 3 strains did not produce hydrogen sulphide when inoculations were made from either culture.

TABLE XIV.

Strain.	Millimeters of lead acetate paper blackened on completion of																	
	18 hours		24 hours		36 hours		48 hours		4 days		5 days		6 days		7 days.		8 days	
	From dextrose peptone culture.	From lactose neutral red culture.	From dextrose peptone culture.	From lactose neutral red culture.	From dextrose peptone culture.	From lactose neutral red culture.	From dextrose peptone culture.	From lactose neutral red culture	From dextrose peptone culture.	From lactose neutral red culture.	From dextrose peptone culture.	From lactose neutral red culture.	From dextrose peptone culture.	From lactose neutral red culture.	From dextrose peptone culture.	From lactose neutral red culture.	From dextrose peptone culture.	From lactose neutral red culture.
No. 1	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	0	2
2	---	---	---	3	---	---	---	---	---	---	---	---	---	---	---	---	0	---
3	---	---	---	1	---	---	---	---	---	---	2	---	---	---	---	---	---	---
4	---	---	---	---	---	---	---	---	1	4	---	---	---	---	---	---	---	---
5	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	0	1
6	---	---	---	---	---	---	---	---	---	5	---	---	---	---	---	---	0	---
7	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	2	0	---
8	---	---	---	---	---	---	---	---	---	---	---	---	---	2	---	---	0	---
9	---	---	---	2	---	---	---	---	---	---	---	---	---	---	---	---	0	---
10	---	---	---	9	12	---	---	---	---	---	---	---	---	---	---	---	---	---
11	---	---	---	---	---	---	---	5	---	---	---	---	---	---	---	---	0	---
12	---	---	---	---	---	---	---	---	---	3	3	---	---	---	---	---	---	---
13	---	---	---	---	---	---	---	---	1	10	---	---	---	---	---	---	---	---
14	---	---	---	---	---	---	---	---	---	---	3	2	---	---	---	---	---	---
15	---	---	---	---	---	---	---	---	---	---	---	---	2	5	---	---	---	---
16	---	---	---	---	---	---	---	---	---	---	4	2	---	---	---	---	---	---
17	---	---	---	---	---	---	---	---	3	11	---	---	---	---	---	---	---	---
18	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	0	0
19	---	---	---	---	---	---	---	---	---	---	2	2	---	---	---	---	---	---
20	---	---	---	---	---	---	---	2	3	---	---	---	---	---	---	---	---	---
21	---	---	---	---	---	---	---	---	---	6	2	---	---	---	---	---	---	---
22	---	---	---	---	---	---	---	---	---	2	---	---	---	---	1	---	---	---
23	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	0	0
24	---	---	---	---	9	---	---	---	---	---	---	---	---	---	---	---	---	0
25	---	---	---	---	---	---	---	---	---	---	3	3	---	---	---	---	---	---
26	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	3	0
27	---	---	---	---	---	---	---	---	---	---	---	3	1	---	---	---	---	---
28	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	0	0
29	---	---	---	---	---	---	---	---	4	10	---	---	---	---	---	---	---	---
30	---	---	---	---	---	---	---	---	2	---	---	2	---	---	---	---	---	---
31	---	---	---	---	---	---	---	---	---	1	3	---	---	---	---	---	---	---
32	---	---	---	---	---	---	---	---	---	---	6	1	---	---	---	---	---	---
33	---	---	---	---	---	---	---	---	---	2	2	---	---	---	---	---	---	---
34	---	4	---	---	---	---	---	---	5	---	---	---	---	---	---	---	---	---
Total number which had produced hydrogen sulphide.	1		5		7		9		26		43		47		49		52	

It should be especially noted that on completion of 24 hours, which has been found to be a sufficient length of time for the production of hydrogen sulphide by the mixed flora of artificial sewage, only 5 of the 68 strains and substrains of the colon organisms tested, had given any hydrogen sulphide at all ; and even after 48 hours, only 9 of the strains had produced hydrogen sulphide.

SUMMARY.

The large amounts of hydrogen sulphide produced by the organisms of artificial sewage are not due primarily to the activities of organisms of the *B. coli* group but to some other group of organisms.

CHAPTER XV.

THE SIGNIFICANCE OF HYDROGEN SULPHIDE PRODUCTION IN THE SANITARY EXAMINATION OF WATER.

It seems unnecessary to discuss in detail in this place the findings and statements of Schardinger or Dunham, as all of the preceding pages have been in a way a discussion of their work or an outgrowth from it. With their statements that the test for hydrogen sulphide producing organisms in water is one which should always be made for detecting the "possibility of infection" in a water sample we are most heartily in accord. While our findings are at variance with theirs as regards some of the details of the method, these differences are sufficiently pointed out in the foregoing chapters and we feel that it would be distinctly out of place to say anything which might tend to belittle the great work of those who were the pioneers in urging the adoption of the test for the bacteria which produce hydrogen sulphide from a sulphur-containing medium, and but for whom, the test might never have been proposed.

We will therefore, merely set down briefly a few of the observations which have been made during the course of this piece of work and which have a bearing on the interpretation of results.

It has been shown in the examination of many samples of water from various sources (see Chapter X) that the method which is proposed will give reliable indications of the presence of the bacteria which produce hydrogen sulphide in from 18 to 24 hours when incubated at 38° C. It has also been shown that waters which are chemically and bacteriologically good will give no hydrogen sulphide production with the proposed medium in even 72 hours.

Only waters which have received sewage pollution from man or domestic animals have given hydrogen sulphide production with the proposed medium. That this hydrogen sulphide is not produced primarily by organisms of the colon group is evident from the work with the 34 isolated strains of organisms of this group. Therefore, it must be produced by some other group of

organisms which are more active proteolytes than the bacteria of the colon group.

According to Macé, [42], the organisms which are most directly concerned in proteolysis are *B. subtilis*, *B. mesentericus*, *B. vulgaris*, *B.* I, II and III of Mouginet[43], *B. fluorescens liquifaciens*, *B. fluorescens putridus*, *B. violaceous*, *Proteus vulgaris* and *Proteus mirabilis*. Any or all of these may be introduced with sewage pollution and may be responsible for hydrogen sulphide production.

An investigation directly bearing on this question has recently appeared by Kendall, Day and Walker, Jour. Amer. Chem. Soc., 35, 1201 (1913), in which they measured the proteolytic activity of a large number of strains of different bacteria by means of the increase in ammonia content of a sugar-free bouillon.

In their article they give various tables, of which the following is a very brief summary of averages arranged in the order of proteolytic activity and also showing whether gas was formed in the different sugars (represented by "g" in the table), or whether the sugars were fermented with acid production, (represented by "x" in the table which follows).

From this table it is evident that *B. proteus vulgaris*, *Vibrio Nassac*, *Sp. cholerae*, and *Sp. Metchnokovi* show very much more proteolytic activity than *B. coli* and hence would produce hydrogen sulphide more rapidly and in larger amounts presumably, while *B. prodigiosus*, *Morgan bacillus* (often mistaken for paratyphoid and closely connected with summer diarrhea), *B. pyocyanus* and *Sp. Finkler and Prior* show slightly more proteolytic activity than *B. coli*.

In the course of the article, the authors state that "the Proteus bacilli are widely distributed in nature where their proteolytic activities are noteworthy in the bacterial degradation of albuminous substances. *B. proteus vulgaris* is the common type. Proteus organisms differ typically from *B. coli* in two noteworthy respects—their inability to produce gas in lactose and their great proteolytic powers. The proteus group is one of the most active chemically met with among the ordinary bacteria. *Cholera vibrios*, causing one of the most dreaded diseases of man, are very active proteolytically, resembling *Proteus bacilli* grossly in this respect."

Organism.	Number of strains investigated.	Average of the maximum increase of NH_3 in mgs. per 100 cc. of broth.	Glucose.	Lactose.	Sucrose.	Manite.
B. proteus vulgaris	8	58.36	g	--	g	g
Vibrio Nassac	1	47.60	x	x	x	x
Sp. cholerae	4	43.93	x	x	x	x
Sp. Metchnikovi	1	41.30	x	--	x	x
B. prodigiosus	1	34.30				
Morgan bacillus	6	29.48	g	--	--	--
B. pyocyanus	2	28.35				
Sp. Finkler and Prior	1	27.30	x	x	x	x
B. coli	13	24.40	g	g	g	g
B. mesentericus	1	23.40	x	--	--	--
Staphylococcus pyogenes	2	22.75	x	x	x	x
B. cloacae	3	21.00	g	g	g	g
B. cuniculicida	9	20.46	x	--	--	x
B. mucosus capsulatus	9	17.27	g	g	g	g
Fowl cholera	2	13.65				
Hog cholera	4	12.43				
Paratyphoid	13	8.29	g	--	--	g
B. lactis aerogenes	5	6.72	g	g	g	g
B. anthracis	3	6.53	x	--	--	--
B. typhosus	9	6.42	x	--	--	x
Mic. melitensis	1	6.30	x	x	x	x
Swine plague	5	4.48	x	--	x	x
B. icteroides	1	4.20				
B. alcaligenes	2	3.50	--	--	--	--
B. diptheriae	11	3.37	x	--	--	--
B. dysenteriae	6	3.27	x	--	--	x(—)
Mic. tetragenus	1	2.10	x	x	x	x
B. alvei	1	1.40	x	x	x	x
Streptococcus pyogenes	1	1.40	x	x	x	x
Mic. zymogenes	1	1.40	x	x	x	x

From the results of the work, already presented, in conjunction with that of Kendall, Day and Walker, cited above, the conclusion may very properly be drawn that when a water under examination gives large amounts of hydrogen sulphide evolved, this hydrogen sulphide is not produced principally by organisms of the *B. coli* group or those having less proteolytic power than

B. coli but by the organisms with greater protelytic power than *B. coli*, none of which, it should be especially noted, give any gas production in lactose media.

Therefore, the test for hydrogen sulphide production is not one which merely supplements the tests made with lactose media. It is much more valuable than that, for it furnishes one of the best means of detecting sewage pollution of water through detecting the presence of the *B. proteus vulgaris* organism and the other energetic proteolytes which are typical of sewage, and it moreover gives an indication of the presence of the much feared *Sp. cholerae*, neither of which functions is performed by the lactose media in use.

SUMMARY.

1. Waters which have received sewage pollution produce hydrogen sulphide from the proposed medium.

2. The hydrogen sulphide is not produced principally by organisms of the *B. coli* group, but by more energetic proteolytes such as *B. proteus vulgaris*, *Sp. cholerae*, *Morgan bacillus* etc., none of which give any gas in lactose media.

3. The test for hydrogen sulphide production is not one which merely supplements the tests made with lactose media. It is more valuable than that for it furnishes one of the best means of detecting sewage pollution by detecting the energetic proteolytes which are ever present in sewage and it gives an indication of the presence of the much feared *Sp. cholerae*, neither of which functions is performed by the lactose media in use.

ORGANISMS WHICH HAVE BEEN REPORTED AS PRODUCING HYDROGEN SULPHIDE.

(a) In one day.
Bacillus typhosus (Eberth and Koch).
" *vulgaris* (Hauser).
Bacterium rhimosclermatis (Trevisan) Migula.
Micrococcus pyogenes aureus (Rosenbach).
Microspira comma (Koch) Schroeter.
(b) In two days.
Bacillus cholerae suis.
" *coli communis* (Emmerich).
" *fluorescens non-liquefaciens.*
" *megaterium.*
" *mirabilis* (Hauser).
Bacterium mallei (Loeffler).
Microspira of Metchnikoff.
(c) In three days.
Bacillus pyocyaneus (Gessard).
Microspira aquatilis (Guenther).
" *of Dunbar.*
(d) In four days.
Bacillus murium (Loeffler).
" *of swine plague.*
Bacterium capsulatum (Sternberg).
Microspira tyrogena (Dencke) Migula.
(e) In six days.
Bacillus Zopfii.
(f) In eight days.
Actinomyces bovis.
(g) In nine days.
Microspira protea (Buchner).
(h) In ten days.
Bacillus fluorescens liquefaciens.
(i) In twelve days.
Bacterium cholerae gallinarum (Perroncito and Toussaint).
(j) In sixteen days.
Microspira saprophile (Weibel).
(k) In twenty-seven days.
Spirillum concentricum.
(l) In thirty days.
Bacillus cyanogenes.
" *prodigiosus.*
" *mesentericus* (Trevisan).

(m) In a variable time.

Bacillus actinobacter.
" *anthracis* (Pollender).
" *cadaveris* (Klein).
" *cavicida.*
" *diphtheriae* (Klebs).
" *foetidus* (Laborius).
" *immobile* (Kruse).
" *janthinus* (Zopf).
" *Kielensis.*
" *lactis aerogenes.*
" *lactis No. 4* (Fluegge).
" *mycoides.*
" *pseudo-butyricus* (Hueppe).
" *rugosus* (Conn).
" *solidus* (Luederitz)
" *sporogenes* (Klein).
" *subtilis.*
" *suipestifer.*
" *suisepticus.*
" *sulphureus* (Holschewnikoff).
" *taveli* (Chester)
" *tetani* (Fluegge).
" *vulgatus* (Trevisan)
" *Wesenbergii* (Chester).

Bacterium citreus (Strassman-Stecker).
" *erythrogenes* (Grotenfelt).
" *erythrogenes vulgatus* (Dyar).
" *pneumoniae* (Zopf).
" *pseudo-diphtheriae.*
" *suicida* (Migula).

Micrococcus coronatus (Fluegge).
" *pyogenes albus* (Rosenbach)

Microspira aestuarii (von Delden).
" *protea* (Buchner).

Mycobacterium diphtheriae (Klebs).
" *rhusiopathiae* (Kitt).

Pseudomonas fluorescens (Fluegge) Migula.
" *ochracea* (Zimmermann).
" *pilocyanea* (Gessard) Migula.

Sarcina fusca (Gruber).
" *lutea* (Fluegge).

Spirillum desulphuricans (Beyerinck).
" *rugula* (Winter).

Tyrothrix urocephalum.

GENERAL SUMMARY.

Chapter III.

1. As that part of peptone which is agglomerated on boiling with water is identical in composition with peptone, and as filtered and unfiltered peptone solutions show no difference in the amount of hydrogen sulphide produced in them, the use of clear, filtered solutions is much to be preferred.

Chapter IV.

1. Irrespective of the inorganic salts present and of the acidity of the medium, a concentration of from 3.0 percent to 4.0 percent of peptone in the inoculated medium is the best for the most rapid and most energetic production of hydrogen sulphide by putrefactive bacteria. It is recommended that 3.0 percent of peptone be used as being more economical and also easier to handle than higher concentrations.

2. Although the addition of beef extract to peptone materially reduced the length of time required for hydrogen sulphide production, the additional labor involved does not appear to be warrented, especially in view of its varying composition.

Chapter V.

1. If sodium chloride is to be used in media for hydrogen sulphide production, not more than 1.5 percent of this salt should be present, as a larger amount has a decidedly inhibiting influence.

2. Cultures to which sodium chloride had been added in concentrations up to 1.5 percent, showed greater production of hydrogen sulphide than was observed in control cultures from which it had been omitted.

3. Sodium sulphate has an inhibiting effect upon hydrogen sulphide production in concentrations greater than 1.5 percent. In concentrations lower than that, it was found to be of slight assistance in augmenting hydrogen sulphide production, being inferior to sodium chloride in that respect.

4. The beneficial influence of potassium chloride on hydrogen sulphide production extends over a *larger range* in concentration than was the case with any other salt tried, no inhibiting action occuring up to a 3.0 percent concentration.

5. In a 3.0 percent unadjusted peptone solution, the presence of from .5 percent to 1.0 percent of potassium chloride had a decidedly beneficial influence and helped to induce *quicker, better and more uniform results than any other inorganic salt tried.*

6. As the concentrations of peptone and of acidity were decreased, the optimum concentration of potassium chloride increased. This would indicate that there is an increasing need of an ionizing salt to make the decreasing amounts of peptone available as food.

7. Magnesium chloride, calcium chloride and ammonium chloride in concentrations up to 2.0 percent had about equal influence upon hydrogen sulphide production, this influence being slightly beneficial.

8. Primary phosphates in concentrations of from 0.0 percent to 1.5 percent not only gave a large amount of turbidity in the culture which is very undesirable but had a decidedly inhibiting influence on hydrogen sulphide production.

9. Secondary phosphates in such concentrations as to furnish .05 percent, .15 percent and .25 percent of the basic elements, showed an undesirable heavy white sediment present; and as compared with the controls, the presence of secondary phosphates had an inhibiting influence on hydrogen sulphide production.

10. Tertiary phosphates had an even greater deleterious influence on hydrogen sulphide production than had primary and secondary phosphates.

11. During the course of the investigation, in which various chlorides, nitrates, sulphates and phosphates were used, many control cultures were prepared containing potassium chloride, sodium chloride and potassium sulphate in a concentration of .5 percent of the inorganic salt, and of these same salts in such concentrations as to furnish .05 percent, .15 percent and .25 percent of the basic elements, and also cultures containing no inorganic salt at all. A careful study and comparison of these check cultures shows that those containing an inorganic salt gave better and quicker results than those without inorganic salt; that the potassium salts were preferable to the sodium salts; that potassium chloride produced the *best* results of any of the salts employed, in that it induced greater and quicker

production of hydrogen sulphide, with greater uniformity of results in duplicate runs and cultures.

12. The presence of calcium carbonate in the cultures was found to be without effect upon hydrogen sulphide production.

13. The presence of primary sodium carbonate had an inhibiting influence on hydrogen sulphide production.

14. The amounts of inorganic salts which would be present in a water under examination are so small as to be negligable.

Chapter VI.

1. Although the effect on hydrogen sulphide evolution produced by the use of different bases and acids in adjusting the initial reaction to phenol phthalein is very slight, potassium hydroxide and sodium hydroxide have been found to be the best bases while hydrochloric acid has been found to be the best acid to use.

Chapter VII.

1. The temperature at which the peptone solutions are titrated has a very decided influence on the reaction when phenol phthalein is used as indicator; the higher the temperature the greater the amount of acidity found.

2. The reaction of a 3.0 percent unadjusted peptone solution, with or without potassium chloride, sodium chloride or potassium sulphate present, was found to be about one-half at 3.0 degrees, two-thirds at 20.0 degrees, and four-fifths at 37.5 degrees of what it was at 95.0 degrees. This is believed to be due to the greater ionization or hydrolysis of peptone solutions at increased temperatures.

3. The reaction of peptone solutions is uneffected by the presence of potassium chloride, sodium chloride or potassium sulphate, but is effected by primary phosphates very materially.

4. The optimum initial reaction of the simple peptone medium, with or without potassium chloride, for hydrogen sulphide production, is from 1.0 percent to 1.5 percent of normal acid, with a slight preference for the higher value. It makes but little difference whether this amount of acidity to phenol phthalein is contributed by an unadjusted peptone solution or whether the peptone is first neutralized with either potassium hydroxide or sodium hydroxide and then made acid with hydrochloric acid.

Chapter VIII.

1. The same variations with temperature were noted after inoculation and incubation of cultures as before.

2. A study of the reactions of the media after inoculation and incubation furnishes excellent corroboration of the conclusions already drawn on the assumption that the amount of acidity produced is a measure of the activity and of the amount of growth and development of the bacterial flora present. From such a study the following deductions may be made :—

(a) With varying amounts of potassium chloride, sodium chloride and potassium sulphate added separately to the media, those media that had received potassium chloride gave the most uniform amounts of increase in acidity.

(b) At the time when hydrogen sulphide was first being produced, an average acidity of about 2.0 percent was found, this acidity increasing with increased production of hydrogen sulphide, until an average acidity near 3.0 percent was reached when most abundant production of hydrogen sulphide was noted.

(c) Cultures containing potassium chloride gave, on the average, larger increases in acidity in both twenty-four and forty-eight hour incubations than did cultures containing either sodium chloride or potassium sulphate, and cultures with any of these salts present gave a larger increase in acidity than cultures containing no added salt.

(d) In the presence of primary phosphates, the development of acidity was very slight.

(e) Tertiary phosphates had an inhibiting effect upon the production of acidity.

(f) The presence of secondary phosphates in some instances led to a greater development of acidity than was noted with peptone alone. This would seem to justify their use in certain classes of culture media.

3. More acid-reacting material is in the sediment of the cultures than in the soluble part.

Chapter IX.

1. The method which has been found to give the most rapid and reliable results is very easy to follow and the medium is simple to make.

Chapter X.

1. Positive results are obtained by the proposed method in 18 hours with sewage polluted waters showing an amount of pollution often met in actual work.

2. No hydrogen sulphide is produced in as long a period of incubation as 72 hours with natural waters of good quality when inoculated into the peptone, potassium chloride medium ; while much hydrogen sulphide is produced in as short a period as 18 hours with natural waters of bad quality, with a nicety of gradation in results corresponding to the quality of the water.

Chapter XI.

1. Of the methods tried, the Liebig-Koch method for total sulphur gave the highest and most consistent results.

2. During the preliminary treatment with nitric acid in the Liebig-Koch method, no sulphur is lost in volatile form.

3. The length of time beyond two hours during which nitric acid is allowed to act has no influence on the Liebig-Koch method.

4. Of the methods for determining a part only of the sulphur, the Schultz method for loosely bound sulphur and the digestion with nitric acid and potassium chlorate for easily oxidized sulphur gave the most consistent and the most valuable results.

Chapter XII.

1. The percent of sulphur present in the very small part of peptone which is insoluble in water is practically the same as in peptone itself. This explains why, in testing for bacteria which produce hydrogen sulphide, it makes no difference whether the medium is filtered or not.

2. The Liebig-Koch method for total sulphur and the potassium chlorate-nitric acid method for easily oxidized sulphur are as accurate for culture media made from peptone and potassium chloride as they are for peptone itself.

3. The ratio of total solids exclusive of potassium chloride to total sulphur is very nearly the same in the soluble and insoluble portions of cultures which have been inoculated with artificial sewage, containing bacteria which produce hydrogen sulphide,

and incubated at 38 degrees for 48 hours, and hence the indications are that the insoluble material is mostly peptone.

4. More material is broken down by the bacteria and more hydrogen sulphide is produced in flasks having much surface of the cultures exposed to air than in flasks having comparatively little surface exposed.

5. It is noteworthy that about 50. percent more total sulphur was converted into hydrogen sulphide when sterile air was passed over the cultures as when they were exposed to quiescent air ; and about 100. percent more total sulphur was, under otherwise identical conditions converted into hydrogen sulphide when sterile air was passed over the cultures as when sterile carbon dioxide was passed over them. Therefore, in the case of the flora which was used, at least, the organisms were most active and produced the most hydrogen sulphide when supplied most freely with air. This fact would appear to be of great importance in connection with work directed toward the elimination of odors arising from septic tanks for sewage disposal.

6. From 25. percent to 30. percent of the total sulphur was converted into hydrogen sulphide when cultures were incubated 48 hours with sterile air passing over the culture, and from 50. percent to 60. percent was thus converted when incubated 72 hours.

7. The volumetric iodine method for the determination of hydrogen sulphide is not available in the analysis of the gases given off by bacteria inoculated into the simple peptone medium, because of the interference of volatile unsaturated organic compounds.

8. Solutions of cadmium chloride containing potassium chloride to prevent colloidal suspension, of sodium peroxide and of potassium hydroxide are available for use in wash bottles through which the gases from bacterial activity may be drawn for the purpose of catching and holding the hydrogen sulphide as metallic sulphide, in which form the sulpur may be determined by the Liebig-Koch method. The use of potassium hydroxide is to be preferred.

9. The sum of the sulphur remaining in the cultures after incubation plus the sulphur in gaseous form, which is practically all present as hydrogen sulphide, equals the total sulphur present

in the cultures before incubation, when the determinations are made by the methods adopted in this investigation.

10. In media made from the part of peptone soluble in alcohol much less sulphur-containing material was broken down and very much less hydrogen sulphide was produced than was found in the media made from the part of peptone insoluble in alcohol, the ratios being about 3:5 and 1:6; while in both of these media, very much less sulphur-containing material was broken down and very much less hydrogen sulphide was produced than in the simple peptone medium, the ratios being respectively about 1:2 and 1:20 in the soluble part of peptone, and 2:3 and 1:4 in the insoluble part.

11. As shown by the percent of total solids exclusive of potassium chloride destroyed, and by the percent of total sulphur converted into hydrogen sulphide, a larger percent of sulphur-containing material than of total peptone was broken down, the ratio being about 3:1. The fact that the bodies of the bacteria were included in the total solids exclusive of potassium chloride, would not materially influence the ratio.

12. A larger percent of easily oxidized sulphur than of total sulphur was converted into hydrogen sulphide by the bacteria, the ratio being about 4:3. It is evident that if a synthetic medium is to be prepared for the detection of the bacteria producing hydrogen sulphide, work upon which is herewith promised, the sulphur should be introduced in an easily oxidized form.

13. A larger percent of loosely bound sulphur than of total sulphur was converted into hydrogen sulphide by the bacteria, the ratio being about 3 : 2.

14. A very slightly greater percent of loosely bound sulphur than of easily oxidized sulphur was converted into hydrogen sulphide by the bacteria, the ratio being about 10 : 9. There was about twice as much easily oxidized sulphur as there was of loosely bound sulphur, both before and after the bacteria had acted upon the medium.

15. It is possible with a fair degree of accuracy to determine the equivalent of hydrogen sulphide for each millimeter of blackening of the lead acetate paper strips in the caps of the special flasks.

Chapter XIII.

1. The feces of the domestic animals contain bacteria which are capable of producing hydrogen sulphide from the simple peptone medium as rapidly and in as large amounts as in the case of the bacteria from human feces.

Chapter XIV.

1. The large amounts of hydrogen sulphide produced by the organisms of artificial sewage are not due primarily to the activities of organisms of the *B. coli* group but to some other group of organisms.

Chapter XV.

1. Waters which have received sewage pollution produce hydrogen sulphide from the proposed medium.

2. The hydrogen sulphide is not produced principally by organisms of the *B. coli* group, but by more energetic proteolytes such as *B. proteus vulgaris*, *Sp. cholerae*, *Morgan bacillus*, etc., none of which give any gas in lactose media.

3. The test for hydrogen sulphide production is not one which merely supplements the tests made with lactose media. It is more valuable than that for it furnishes one of the best means of detecting sewage pollution, by detecting the energetic proteolytes which are ever present in sewage and it gives an indication of the presence of the much feared *Sp. cholerae*, neither of which functions is performed by the lactose media in use.

Note.—Some preliminary work, performed in the laboratory while this article has been in press, indicates very strongly that hydrogen sulphide is more rapidly produced by a mixed culture, in which several organisms are growing together, than by pure cultures. More work on this subject is promised.

BIBLIOGRAPHY.

1. Gayon, Compt. rend., Acad., 85, 1074 (1877).
2. Miquel, Bull. soc. Chim., (2), 32, 127 (1879).
3. Miquel, Ann. de l'observatoire de Mont-Souri (1880).
4. Boehn, Monatsh., 3, 224 (1882).
5. Fischer, Dingl. polyt. J., 247, 501 (1883).
6. Ricket, Compt. rend., 97, 1004 (1884).
7. Miller, Deut. med. Wochschr., 49, (1885).
8. Hoppe-Seyler, Z. physiol. Chem., 10, 432 (1886).
9. Hoertling, Dissert. Berlin, (1886).
10. Winogradsky, Botan. Zeitung, 31, 37 (1887).
11. Mueller, Berl. klin. Wochschr., 23, 405 (1887).
12. Mueller, Berl. klin. Wochschr., 24, 436 (1887).
13. Rosenheim, Deut. med. Wochschr., 24, 530 (1887).
14. Rosenheim, Fortschritte med., 5, 345 (1887).
15. Salkowski, Berl. klin. Wochschr., 25, 722 (1888).
16. Schrank, Wiener medicinische Jahrbucher, 303, (1888).
17. Holschewnikoff, Fortschritte med., 7, 201 (1889).
18. Fromme, Dissert. Marburg (1891).
19. Debraye and Legrain, Compt. rend. soc. biol., (9) 11, 466.
20. Stagnitta and Balisteri, Arch. Hyg., XVI, 10, (1893).
21. Petri and Maasen, Arb. kais. Gesundh., VII, 119 (1892).
22. Petri and Maasen, Arb. kais. Gesundh., VIII, 318 (1893).
23. Petri and Maasen, Arb. kais. Gesundh., IX, 490 (1894).
24. Rubner, Arch. Hyg., XVI, 53 (1893).
25. Rubner, Arch. Hyg., XVI, 78 (1893).
26. Schardinger, Cent. Bak., 16, 853 (1894).
27. Karplus, Arch. path. Anat., CXXXI, 2, 210 (1894).
28. Beyerinck, Centr. Bakt. Parasitenk., I, 1, (1895).
29. Zelinsky, Fortschritte russ. chem. phys. Gesel., XXV, 5 (1893).
30. Beyerinck, Centr. Bakt. Parasitenk., I, 49 (1895).
31. Beyerinck, Centr. Bakt. Parasitenk., I, 104 (1895).
32. Beyerinck, Arch. neerland. sci. exact. nat., 233 (1895).
33. Orlowski, J. med. militare Russie (1895).
34. Dunham, J. Am. Chem. Soc., XIX, 591 (1897).
35. Morris, Arch. Hyg., 30, 304 (1897).
36. Selensky and Brussilowsky, C. V. Odessa (1898).
37. Beyerinck, Arch. neerland. sci. exact. nat., II, 4, 1 (1900).
38. Saltet, Centr. Bakt. Parasitenk., II, 6, 695 (1900).
39. Winkler, Z. anal. Chem., 40, 772 (1901).
40. von Delden, Centr. Bakt. Parasitenk., II, 11, 81 (1903).
41. von Delden, Centr. Bakt. Parasitenk., II, 11, 113 (1903).
42. Mace, Traite Pratique de Bacteriologie, Paris (1904)
43. Mouginet, Quelques Bacteries des putrefactions, Nancy (1890).
44. Beyerinck, Arch. neerland. sci, exact. nat., II, 9, 131 (1904).

45. Rank, Dissert. Zurich (1907).
46. Huss, Centr. Bakt. Parasitenk., 59 (1907).
47. Hildebrandt, Beitr. chem. Physiol., (Hofmeister) 11, 409 (1908).
48. Belonowski, Biochem. Z., 6, 251 (1908).
49. Paus, Centr. Bakt. Parasitenk., 45, 81 (1908).
50. Nawiaski, Arch. Hyg., 66, 209 (1908).
51. Kestler, J. biol. Chem., 3, 35 (1908).
52. Porcher and Pamisset, Compt. rend. soc. biol., 68, 653 (1909).
53. Herter, Trans. Assoc. Am. Physicians, 23, 246 (1909).
54. Brasch, Biochem. Z., 18, 380 (1909).
55. Dunschmann, Compt. rend., 146, 999 (1909).
56. McCrudden, J. biol. chem., VIII, 109 (1910).
57. Kendall, J. biol. chem., VI, 499 (1908).
58. Wiechardt, Zentr. ges. Physiol. Path. Stoffw., 5, 131 (1910).
59. Lange and Pippe, Arb. kais. Gesundh., 33, I, 127 (1910).
60. Kuehel, Pharm. Zeit., 56, No. 61, 616 (1911).
61. Bainbridge, J. Hyg., 11, 341 (1911).
62. Neuberg, Biochem. Z., 37, 501 (1911).
154. Kendall, Day and Walker, J. Am. Chem. Soc., 35, 1201 (1913).

REFERENCES ON THE CHEMICAL COMPOSITION OF PEPTONE.

63. Kossel, Deut. chem. Gesel. Ber., 12, 703.
64. Herth, Akad. Ber., (11), 76, 869.
65. Palm, Zeit. anal. chem., 27, 359.
66. Koenig and Kisch, Zeit. anal. chem., 28, 191.
67. Krueger, Pflueger's Archiv., 43, 244.
68. Demayer, Chem. Centr., I, 1084 (1890).
69. Bruylants, Revue Internal. Scientif., 3, 167.
70. Heaton and Vasey, Analyst, 17, 28.
71. Schuetzenberger, Compt. rend., 115, 208.
72. Ciamician and Zanetti, Gaz. chim. ital., 22, 449.
73. Schroetter, Monatsh., 16, 609 (1895).
74. Pick, Zeit. physiol. chem., 24, 246 (1897).
75. Schroetter, Monatsh., 19, 211 (1898).
76. Schroetter, Zeit. physiol. chem., 26, 338 (1898).
77. Hopkins and Pinkus, Ber , 31, 1311 (1898).
78. Folin, Zeit. physiol. chem., 25, 152 (1898).
79. Sollmann, Amer. J. Physiol., 7, 203 (1902).
80. Siegfried, Zeit. physiol. chem., 38, 259 (1903).
81. Levene and Van Slyke, Biochem. Z., 13, 440.
82. Lematte and Saves, Compt. rend., 148, 553.
83. Henriques and Gjaldbaek, Zeit. physiol. chem., 67, 8 (1909).
84. Handovsky, Z. Chem. Ind. Kalloide, 7, 183.
85. Pepin, Bull. sci. pharmacolog., 17, 594.

REFERENCES ON THE EFFECT OF INORGANIC SALTS IN MEDIA.

86. Forster, R. Acad. Science, Amsterdam, April 1890.
87. Frankland, Proc. Roy. Soc., LVI, 535.

88. Pringle, Acad. des Sciences, 1750.
89. Rideal, Disinfection and Preservation of Food, page 98.
90. Thresh and Porter, Preservatives in Food, page 13.
91. Cameron and Robinson, J. Physic. Chem., 11, 577.
92. Buch, Z. anorg. chem., 52, 325 (1907).
93. Stoklasa, Z. landw. Vers. Wes. Oesterr., 11, 52.
94. Russo, Compt. rend. soc. biol., 68, 716 (1909).
95. Frouin, Compt. rend. soc. biol., 68, 801 (1909).
96. Kindborg, Berl. klin. Wochschr., 40, 1800.
97. Huene, Biochem. Centr., 8, 151.
98. Koelker, J. biol. chem., 175 (1910).
99. Thies, Mitth. Grenzgeb., 21, 239.
100. Krzemieniewska, Anz. akad. Wiss. Krakau, (B), 376 (1910).
101. Lipman, Bot. Gaz., 47, 41.
102. Weichel, Arb. kais. Gesundh., 34, 247.
103. Lipman, Bot. Gaz., 49, 207.
104. Calcaterra, Ann. Inst. Maragliano, 4, 169.

REFERENCES ON THE DETERMINATION OF SULPHUR IN ORGANIC SUBSTANCES.

105. Mixture, Chem. News, 46, 217.
106. Keiser, Amer. Chem. J., 5, 207.
107. Hammersten, Zeit. physiol. Chem., 9, 273.
108. Klobukoff, Ber., 18, 1861.
109. Klason, Ber., 19, 1910.
110. Kochs, Chem. Centr., 1886, 894.
111. Beringer, Chem. News, 59, 41.
112. Messinger, Ber., 21, 2910.
113. Burton, Amer. Chem. J., II, 472.
114. Prunier, Compt. rend., 109, 904.
115. Hempel, Zeit. angew. chem., 1892, 393.
116. Angeli, Gaz. cim. ital., 21, I, 163.
117. Mohr, Zeit. physiol. chem., 20, 403.
118. Duering, Zeit. physiol. chem., 22, 281.
119. Schulz, Zeit. physiol. chem., 25, 16.
120. Osborne, Zeit. anal. chem., 1902, 41, 25.
121. Dobriner, Zeit. anal. chem., 40, 44.
122. Osborne, J. Am. Chem. Soc., 24, 140.
123. Fraps, J. Am. Chem. Soc., 24, 346.
124. Beistle, J. Am. Chem. Soc., 24, 1093.
125. Sherman, J. Am. Chem. Soc., 24, 1100.
126. Petersen, Zeit. anal. chem., 42, 406.
127. Modrakowski, Zeit. physiol. chem., 38, 562.
128. Barlow, J. Landw., 51, 289.
129. von Konek, Zeit. angew. chem., 16, 516.
130. Krummacher, Zeit. Biol., 45, 310.
131. Le Clerc and Dubois, J. Am. Chem. Soc., 26, 1808.

132. Neumann and Meinertz, Zeit. physiol. chem., 43, 37.
133. Dubois, J. Am. Chem. Soc., 27, 729.
134. Sadtler, J. Am. Chem. Soc., 27, 1888.
135. Berger, Compt. rend., 143, 1160.
136. Folin, J. Biol. Chem., 1, 131.
137. Bay, Compt. rend., 146, 333.
138. Rupp, Chem. Zeit., 32, 984.
139. Koch, J. Am. Chem. Soc., 31, 1329.
140. Folin, J. Am. Chem. Soc., 31, 284.
141. Grindley, J. Am. Chem. Soc., 31, 52.
142. Benedict, J. biol. chem., 7, 101.
143. Denis, J. biol. chem., 7, 5, 401.
144. Schreiber, J. Am. Chem. Soc., 32, 977.
155. Folin, J. biol. chem., 3, 81 (1907)
156. Parr, J. Am. Chem. Soc., 30, 764 (1908).
157. Sherman, Organic Analysis, MacMillan Co., 295 (1912).

REFERENCES ON EFFECT OF REACTION OF MEDIA ON BACTERIA.

145. Carapelle, Centr. Bakt. Parasitenk., 47, 549.
146. Lazarus, Compt. rend., 149, 423.

MISCELLANEOUS REFERENCES APPLICABLE TO THIS PROBLEM.

147. Weigl, Nachr. Ges. Wiss., Goeitengen, 525 (1906).
148. Padoa and Cambi, Atti. accad. Lincei (V), 787.
149. Baubigny, Compt. rend., 143, 678.
150. Padoa and Cambi, Gaz. chim. ital., 37, II, 111.
151. Reitz, Z. angew. chem., 22, 100.
152. Maasen and Schoenewald, Mitt. K. Biol. Anast. Land. u. Forstw., 32 (1910).
153. Moore and Williams, Biochem. J., 5, 181.

CUT NO 1

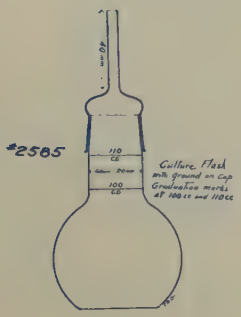


PLATE A

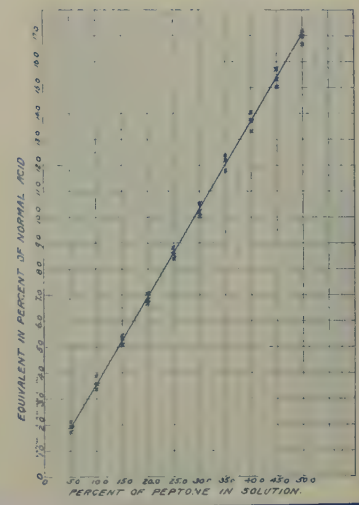


PLATE B.

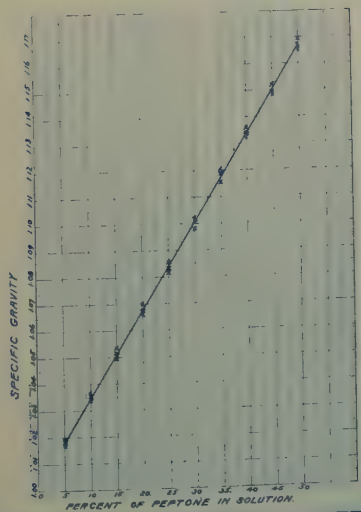
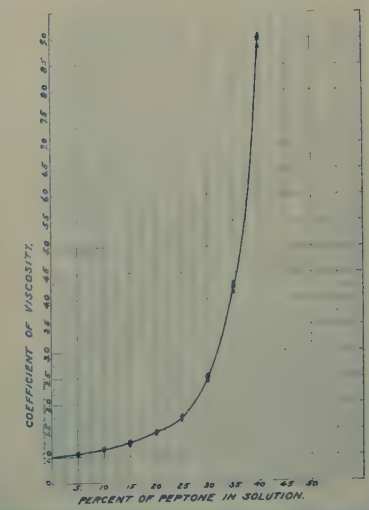
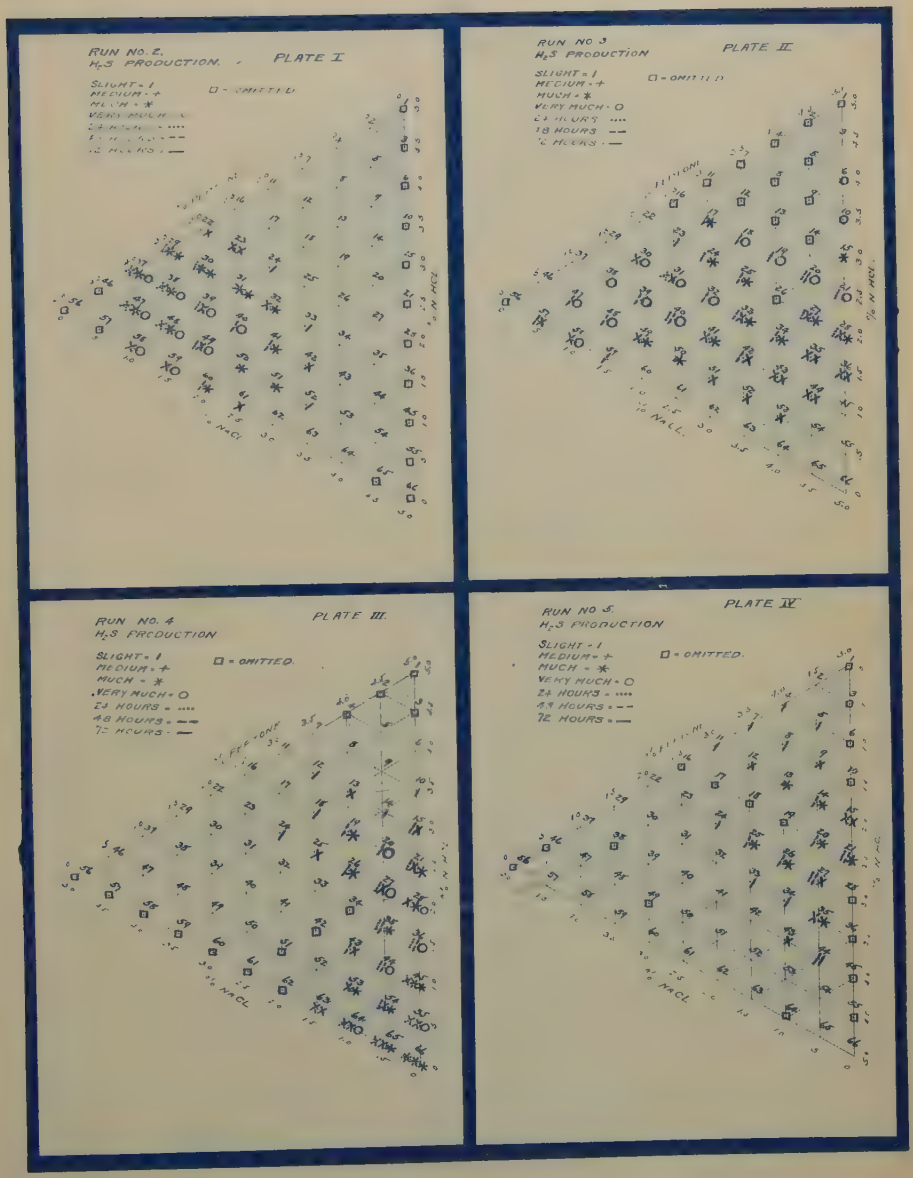
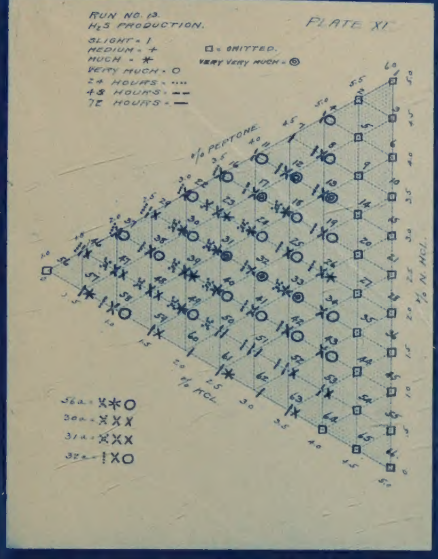


PLATE C.




RUN NO. 2.
H₂S PRODUCTION.
PLATE I
SLIGHT = 1
MEDIUM = +
MUCH = *
VERY MUCH = O
24 HOURS = ...
48 HOURS = ---
72 HOURS = —
□ = OMITTED.
RUN NO 3
H₂S PRODUCTION
PLATE II
SLIGHT = 1
MEDIUM = +
MUCH = *
VERY MUCH = O
24 HOURS ....
48 HOURS ---
72 HOURS —
□ = OMITTED.
RUN NO. 4
H₂S PRODUCTION
PLATE III.
SLIGHT = 1
MEDIUM = +
MUCH = *
VERY MUCH = O
24 HOURS = ...
48 HOURS = ---
72 HOURS = —
□ = OMITTED.
RUN NO 5.
H₂S PRODUCTION
PLATE IV
SLIGHT = 1
MEDIUM = +
MUCH = *
VERY MUCH = O
24 HOURS = ...
48 HOURS = ---
72 HOURS = —
□ = OMITTED.


RUN NO. 13.
H_2S PRODUCTION.
PLATE XI
SLIGHT - I
MEDIUM - +
MUCH - *
VERY MUCH - O
24 HOURS - ....
48 HOURS - ——
72 HOURS - ——
□ = OMITTED.
VERY VERY MUCH - ◎
% PEPTONE
% NACL
% N. ACID
36a = X*O
36b = XXX
31a = X*XX
32a = IXO

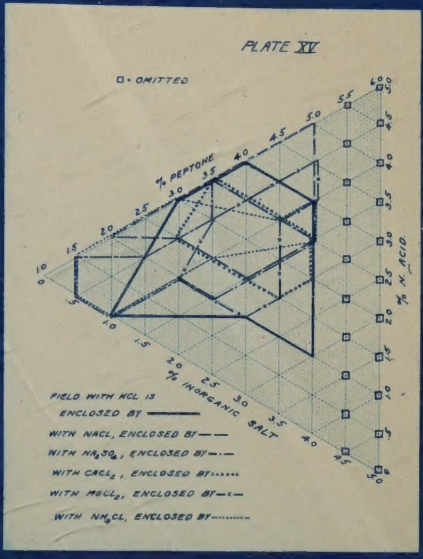


PLATE XV
□ = OMITTED
% PEPTONE
% INORGANIC SALT
% N. ACID
FIELD WITH HCL IS
ENCLOSED BY ——
WITH NACL, ENCLOSED BY——
WITH Na_2SO_4, ENCLOSED BY—·—
WITH $CaCl_2$, ENCLOSED BY·····
WITH $MgCl_2$, ENCLOSED BY—··—
WITH NH_4Cl, ENCLOSED BY·····

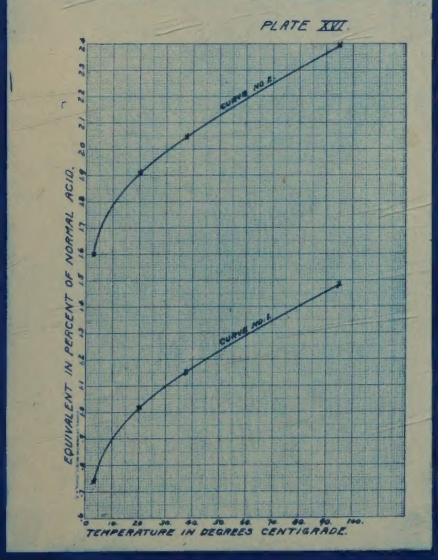


PLATE XVI
CURVE NO. 2.
CURVE NO. 1.
EQUIVALENT IN PERCENT OF NORMAL ACID.
TEMPERATURE IN DEGREES CENTIGRADE.

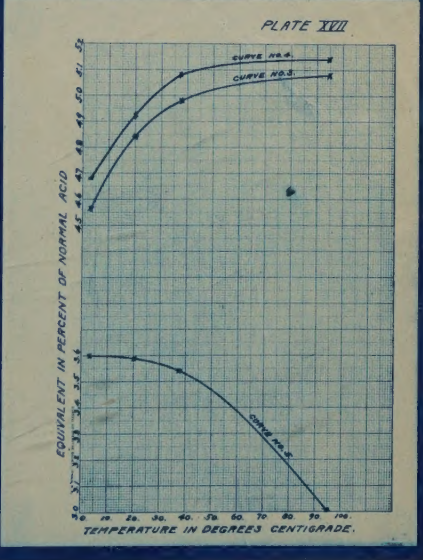


PLATE XVII
CURVE NO. 4.
CURVE NO. 3.
CURVE NO. 5.
EQUIVALENT IN PERCENT OF NORMAL ACID
TEMPERATURE IN DEGREES CENTIGRADE.